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THE PRESERVATIVE EFFECT OF SOME REDUCING SYSTEMS ON THE VIRUS OF TOMATO SPOTTED WILT[1]

by

RUPERT J. BEST

(From the Waite Agricultural Research Institute, University of Adelaide, South Australia).

(Submitted for publication 5th November, 1938.)

INTRODUCTION.

Suspensions of the virus of tomato spotted wilt (T.S.W.) in buffer solutions at pH values favouring virus activity become non-infectious after a few hours' exposure to air at room temperatures. In a previous paper (Best and Samuel, 1936) the effects of a number of reducing agents in prolonging the period of activity of the inocula under controlled conditions at pH 7 were described. The preservative effect on the virus of some of the systems there described has since been studied over longer time periods, and some previously untried systems such as ascorbic acid, glutathione, thioglycollate and adrenaline have also been tested. The results of these tests are recorded in the present paper.

EXPERIMENTAL METHODS.

Methods of preparing and handling inocula were essentially the same as those described in previous papers of this series. A modified Holmes-Samuel primary lesion method, using *Nicotiana tabacum* (var. Blue Pryor) as test plant, was used to estimate relative concentrations of active virus (Best, 1937). The initial concentration of infective juice was chosen as 1 in 100 in order that any fall in lesion numbers could (within limits) be interpreted as a proportionate fall in active virus units.

All solutions were made up in a composite phthalate-phosphate-borate buffer (0·04 M) of pH 7. In cases where the test substance altered the pH value of the solution this was adjusted to pH 7 by the addition of NaOH or HCl before adding the virus.

To determine redox potentials, platinum gauze electrodes were used in an atmosphere of nitrogen free from oxygen. The nitrogen was purified as previously described (Best and Samuel, 1936).

Unless otherwise stated the test plants were transferred to the laboratory 24 hours before being inoculated, and the lesions were counted after the rate of their appearance had slowed down and before their spreading rendered counting difficult.

[1] Glasshouse facilities and part of the cost of these investigations are being provided by the Council for Scientific and Industrial Research.

EXPERIMENTAL RESULTS.

Platinised Platinum-Hydrogen System.

It had been found (Best and Samuel, 1936) that when infective juice was added to a buffer solution (pH 7) containing platinised platinum gauze and through which pure hydrogen was passed, the concentration of active virus remained unchanged over the four-hour period of the experiment, whereas the control suspension had become inactive in that time.

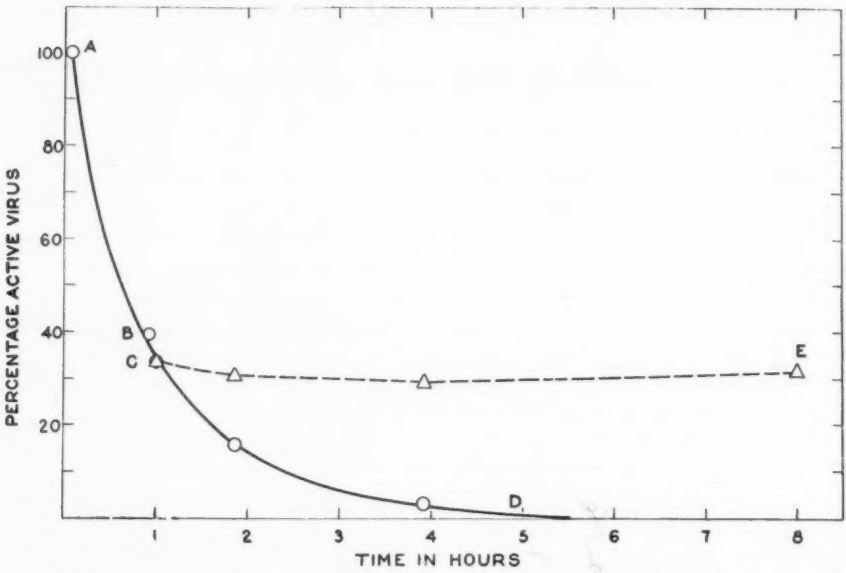

Figure 1. Illustrating (1) the rate of inactivation of a suspension of tomato spotted wilt virus (buffered at pH 7) when exposed to air (A, B, C, D) and (2) the arresting of the inactivation process when a portion was transferred to a container through which hydrogen was bubbling in the presence of platinised platinum gauze (C, E). [Juice] = 1/100. Temperature 21°C.

The experiment now to be described was designed to determine whether the normal oxidative inactivation of the virus could be arrested by the above treatment after it had begun, and to test the possibility of reactivating virus which had been inactivated during exposure to air.

A suspension of the virus was prepared by adding 1 ml. of freshly-expressed infective juice to 99 ml. of buffer solution (pH 7). The suspension was used as the control and was exposed to air. Five minutes after adding the juice a portion

was withdrawn and was inoculated on the appropriate leaves of two previously arranged 5 x 5 Latin squares. After 56 minutes' exposure to air another portion was withdrawn and was inoculated on one half of each of the appropriate leaves of the same squares (this inoculation took three minutes to perform).

Immediately after this inoculation (60 minutes from the beginning of the experiment) one-half of the remaining control suspension was transferred to a flask through which pure hydrogen was passing, and which contained platinum gauze which had been coated electrolytically with platinum black. This was now designated the "test" suspension and was inoculated on the opposite halves of the same leaves as the 56-minute control inoculum had been. The test inoculum (under hydrogen) and the control inoculum (exposed to air) were again inoculated against each other on opposite halves of the appropriate leaves after lapses of 52 minutes, 2 hours 55 minutes, and 7 hours. During the inoculation process the inocula were stored in dropping funnels under an atmosphere of hydrogen. Inoculum was dropped from the funnels on to the ground glass spatulas as required.

TABLE 1.

Showing the Protective Effect on a Suspension of the Virus of Tomato Spotted Wilt in Buffer Solution at pH 7, of Hydrogen in the Presence of Platinised Platinum.

[Juice] of Inocula = 1/100. Temperature, 21° C.

C = Control inoculum exposed to air.

T = Test inoculum protected by the H_2–Pt system.

Time.	5 minutes.		56 mins.	60 mins.	1 hr.	52 mins.	3 hrs.	55 mins.	8 hrs.	
Inoculum	C	C	C	T	C	T	C	T	C	T
No. of lesions on 10 half leaves	137	147	56	48	15	44	5	42	0	46
	n.s.		n.s.		**		**		**	
	**		n.s.		n.s.		n.s.		n.s.	
Per cent of control (mean initial value 142)	100		39·5	33·8	10·6	31·0	3·5	29·6	0	32·4

n.s. = no significant difference.

* = difference is significant at the 0·02 probability level.

** = difference is significant at the 0·01 probability level.

This experiment was performed with two 5 × 5 Latin squares on 25 tobacco plants trimmed to two leaves per plant.

The results of the subsequent lesion count are summarized in Table 1 and Fig. 1. From these it is clear that although rapid inactivation of the virus occurred in the suspension exposed to air no significant changes took place in the activity of the portion which was subjected to the reducing conditions of the hydrogen-platinum system.

Long Range Storage Trials with Cystein as Preservative (Oxygen Excluded).

That virus activity may be preserved *in vitro* for at least 48 hours by the presence of cystein in T.S.W. inocula, provided the pH value is also favourable, was shown in a previous paper (Best and Samuel, 1936). It was of some importance for its own sake and also from the point of view of keeping the virus active during attempts to purify it, to determine the length of time it was possible to maintain the virus in an active state.

Trial No. 1.

The freshly expressed juice of infected tomato plants was used to prepare two suspensions, one (the control) in 0·04 M composite buffer solution of pH 7·5 and another (the test) in a similar solution but 0·01 M in respect to cystein.[2] The concentration of infective juice was 1 in 100. Pure hydrogen was bubbled continuously through both suspensions. Purification of the hydrogen and precautions to exclude oxygen were as previously described (Best and Samuel, 1936). At suitable intervals over a period of six days, portions of the inocula were withdrawn (through a tap at the bottom of the specially designed vessels) and were inoculated against each other on opposite halves of 20 tobacco leaves. The temperature of the inocula was maintained at about 0°C. by a packing of crushed ice.[3]

The 80 test plants required for inoculation purposes were drawn from a single potting of 100 tobacco plants. These were divided into 8 groups of 10 plants per group. Actually the test plants which received the last inoculum were 6 days older than the first group were at the time of inoculation. To obtain some sort of check on a possible susceptibility gradient with age within this main batch, the last inoculum was also applied to a group of 10 plants (8B) from a batch which had been sown and potted a week later than the main batch. The plants of group 8B would, therefore, be more nearly comparable (in respect to their condition) with the plants which received the first inoculum than the plants of the last group (8A) of the main batch would be. Each group of plants was trimmed to two leaves per plant and was transferred to the laboratory 40 hours before the plants were inoculated. The main difficulty in carrying out tests of this nature over periods which extend beyond 2 or 3 days is the liability of the test plants to undergo changes in suceptibility. As shown previously (Samuel, Best and Bald, 1935) variability in the susceptibility of plants at the moment of inoculation is reduced by the above procedure. It has also been shown (Best, 1936) that the conditions of light and temperature to which test plants are exposed, after they have been inoculated with one and the same suspension of virus, affect the number of lesions which finally develop. Any possible differential effects due to these causes were reduced as far as possible by keeping the inoculated plants in the laboratory for 48 hours before transferring them back to the glasshouse. By this means the conditions in respect to temperature and light were made very uniform for all groups during the early stages of lesion formation. These favourable conditions were further enhanced by the chance that a period of cool, cloudy weather (autumn of 1935) set in at the beginning of the experiment and continued to the end, thus making conditions in the glasshouse also more uniform than usual.

Lesion counts were made daily after a reasonable number had appeared. Each group was counted on at least four successive days. In all cases the count recorded for the seventh day after

[2] The cystein used was a particularly pure sample of the hydrochloride prepared from Australian merino wool by Mr. H. R. Marston, of the C.S.I.R.'s Division of Animal Nutrition, to whom my thanks are due.

[3] This required the attendance of some one during the whole of the six day period to renew the ice packing every few hours. My thanks are due to Mr. V. A. Stephen for taking his share of these long vigils.

inoculation was taken as the basis of comparison as at this stage the rate of increase had slowed down considerably and was optimal for the purpose in hand. The results are summarized in Table 2.

TABLE 2.

Showing the Activity of Suspensions of the Virus of Tomato Spotted Wilt over a Period of Six Days. [Juice] = 1/100. Temperature of Inocula = 0° C.

Control suspension in buffer solution.
Test suspension in buffer solution 0·01 M. in respect to cystein.
Both suspensions had an initial pH value of 7·5 which changed very little during the course of the experiment.
Pure hydrogen was bubbled continuously through both suspensions.

Time stored (in hours)	1	12	24	48	72	96	120	144	
Plant Group No.	1	2	3	4	5	6	7	8A	8B
Tests for significance between controls			n.s.	n.s.	**	**	**	**	**
Lesions on 20 half-leaves: Control	401	512	321	369	159	20	3	0	0
Test	407	654	404	516	449	264	274	315	512
Tests for significance between tests			n.s.	n.s.	n.s.	**	**	n.s.	n.s.

n.s. = no significant difference.
* = difference is significant at the 0·02 probability level.
** = difference is significant at the 0·01 probability level.

In addition to the two suspensions described in the foregoing, two other suspensions were prepared at the same time from the same stock of infective juice. One of these (No. 3) was a duplicate of the cystein test suspension but it was kept in total darkness and was left undisturbed for 6 days. The other (No. 4) was a suspension in 0·01 M solution of sodium sulphite buffered at pH 7·5. These suspensions were also kept at 0°C. and in an atmosphere of hydrogen. After 144 hours' storage each of these suspensions was inoculated on five two-leaved tobacco plants drawn from the same batch as group 8B. The former suspension had produced (seven days after inoculation) 447 lesions and the latter 90 lesions on twenty half-leaf replicates.

Reference to Table 2 reveals that after 48 hours the virus in the control suspension was not significantly different from its original value, but thereafter a significant fall in active virus concentration took place. After five days' storage the proportion of active virus had been reduced to less than 1 p.c. of its original value, and by the sixth day the suspension was non-infective. Under the circumstances, the number of lesions produced by the test suspension remained remarkably constant over the whole of the six day period. There was no significant difference between the lesion numbers of the first and final inoculations. The number of lesions from the fourth and fifth day inoculations was in each case significantly lower than that from the first inoculation, but since there was no significant difference between the numbers for the first and last inoculations the above differences

appear to be due to a lower susceptibility of the test plants. This deduction is strengthened by the fact that the number of lesions on group 8B was significantly higher than that on group 8A, although all plants in these groups were treated with the same inoculum (all inocula were protected against inactivation during inoculation and the plants of these two groups were alternated during the inoculation procedure). It is clear that the plants of group 8A had a significantly lower susceptibility than the plants of group 8B. The number of lesions on group 8B was actually higher than that on the group which had received the first inoculum six days previously, and, although this difference is shown as not significant in Table 2, it was nearly significant. In view of these considerations *we may conclude that the concentration of active virus was as high after six days' storage as it was at the beginning of the experiment.*

An interesting point brought out by the table is that the number of lesions at the 12-hour inoculation was significantly higher than those at the 1-hour and 24-hour inoculations. This applies to both test and control suspensions, although the increase in the number over that of the first inoculation is proportionately higher for the test inoculum. The inoculation after 12 hours' storage was performed at night (9.40 p.m., mean inoculation time). All other inoculations were performed during the morning (9.40 a.m., mean inoculation time). On a number of other occasions when a series of inoculations of a protected suspension of the virus has included an inoculation performed at night the same phenomenon of a rise in lesion numbers has been observed. A parallel case is provided by another experiment recorded in this paper (Table 7, page 11) where the final inoculation was performed at night. Increases which are probably due to changes in the degree of dispersion of the virus do sometimes take place during the early stages of an experiment, but these have reached an end point in less than one hour, and it was to eliminate this effect that the first inoculation was performed one hour after adding the juice to the media. In other experiments not reported here the lesions number has remained constant for several inoculations performed at intervals during the day, and has then increased suddenly for the night inoculation. We may therefore rule out any possible dispersion effect and ascribe the increase to a more favourable environment for virus multiplication at night. It would be difficult to determine how much of this would be due to changes in the internal and how much to changes in the external environment.

The inhibitory effect of daylight on the development of T.S.W. lesions (Best, 1936) is a factor which must be taken into account. Although this is primarily connected with the external environment its ultimate effects are concerned with the internal environment. This effect has been discussed in the paper referred to (p. 236–237) and similar explanations may be applied to the present case. There are also possibilities of differences in the pH value as well as oxygen tension of plant cells (or their contents) during the dark as opposed to the light period.

The number of lesions produced by suspension No. 3 from which light was

excluded was not significantly different, after six days' storage, from the corresponding suspension exposed to light. There was therefore no inactivation of virus *in vitro* by daylight of laboratory intensity.

Although the virus in suspension No. 4 which contained sodium sulphite as a preservative was still active after six days, its concentration had been reduced to about 20 p.c. of its original value.

Trial No. 2.

Having maintained the virus in an active state for six days an experiment was designed to determine how long the virus would remain active in sealed tubes. A suspension of infective juice was made up in a phthalate-phosphate-borate buffer solution of pH 7·5 containing cystein (0·01 M) exactly as described for the previous experiment. Pure hydrogen was bubbled through the suspension for four hours. Ten-millilitre aliquots were then transferred to specially constructed Pyrex tubes through which pure hydrogen was passing. The tubes were sealed off and stored in a refrigerator whose temperature varied little from 1°C. Three hours from the beginning of the experiment the first tube was opened, the suspension was transferred to a dropping funnel through which hydrogen was passing and was then inoculated on the leaves of ten tobacco plants which had been trimmed to two leaves per plant. These plants had been transferred to the laboratory thirty hours before being inoculated and were taken back to the glasshouse forty-two hours after being inoculated. They were stored in a compartment which had been fitted with calico shades to keep off direct sunlight. Other tubes were opened at suitable intervals over a period of forty-two days and the contents inoculated on batches of ten tobacco plants treated in the same way as the first batch. The lesions produced were counted on three successive days (for each batch) and the counts recorded in Table 3 are, in all cases, those made six days after inoculation. The twenty-eight day and thirty-five day inoculations were made on two batches of plants differing in age by one week and for the thirty-five day inoculation the contents of two tubes were bulked before inoculation.

TABLE 3.

Longevity of the Virus of Tomato Spotted Wilt in Sealed Tubes in a Suspension Buffered at pH 7·5 and 0·01 M in respect to Cystein. The space above the Suspension was taken up by Pure Hydrogen. The Temperature of Storage was about 1° C. [Juice] = 1/100.

Time stored.	3 hours.	7 days.	15 days.	21 days.	28 days.		35 days.		42 days.
Age of test plants in weeks	6	5	6	5	5	6	5	6	5
No. of lesions on 40 half-leaves	1,393	1,144	665	154	8	91	1,025	690	0
		*	**		**		**		
		n.s.							

n.s. = no significant difference.
* = difference is significant at the 0·02 probability level.
** = difference is significant at the 0·01 probability level.

The virus was still quite active after thirty-five days' storage, but was inactive after the forty-second day. It is difficult to form a reliable estimate of the quantitative relationships of the various inocula in respect to active virus content, because of the variation from week to week of the susceptibility of the host plants

and the differences in temperature and light intensity prevailing at the time of lesion development.

Even where these latter conditions were the same, as for the plants at the 28-day and at the 35-day inoculations, groups of plants differing in age by only one week reacted very differently in respect to the number of lesions produced by the same inoculum. Of the two groups inoculated on the twenty-eighth day, the six-weeks-old plants developed more than eleven times as many lesions as the five-weeks-old plants, although both groups had received the same inoculum. For the thirty-five-day inoculation the five-weeks-old plants developed nearly twice as many lesions as the six-weeks-old plants. These differences which were clearly due to differences in susceptibility of the test plants, were significant in both cases.

The number of lesions produced by the first inoculation was significantly higher than that produced by any of the subsequent inoculations. In view of the regular falling-off in lesion numbers during the first twenty-eight days, and the fact that after forty-two days the suspension was non-infective, we may conclude that there was a falling-off in the concentration of active virus. It is interesting to note, however, that the number of lesions produced by the thirty-five-day inoculation was not significantly different from that produced by the seven-day inoculation, was significantly lower than that produced by the first inoculation, and was significantly higher than those produced by the fifteen-day, twenty-one-day and twenty-eight-day inoculations. Either the falling-off in virus activity was greater in some tubes than in others or the test plants varied greatly in susceptibility. The former is very unlikely, and we may conclude that after thirty-five days' storage the concentration of active virus has fallen significantly but not very greatly, but that after forty-two days' storage the inoculum was no longer infective. The maintaining of virus in an active state for thirty-five days represents a great advance when it is remembered that the virus in unprotected suspensions in buffer solution alone becomes non-infective in less than twelve hours.

Glutathione and Thioglycollic Acid as Protective Agents.

In the experiments just described in which cystein was used as a preservative, oxygen was excluded. However, cystein will protect the virus against inactivation even when suspensions are exposed to air (Best and Samuel, 1936), although in this case the protective action does not last so long. It was of some interest to test the effect of other substances whose reducing properties were centred in sulphydryl groups. Glutathione and thioglycollic acid were two such substances which were available. The results of tests carried out with these two substances are summarized in Tables 4 and 5.

In both cases the test substance was dissolved in 0·04 M buffer solution of pH 7 to give a 0·01 M solution. Sufficient NaOH solution was then added to neutralize the acidity of the substances and bring the pH value back to 7 before adding the infective juice. In accordance with the usual practice, test and control suspensions were in both cases divided into as many aliquots as there were to be time-inoculations. All suspensions were exposed to air throughout.

TABLE 4.

Showing the Preservative Effect on the Virus of Tomato Spotted Wilt of Reduced Glutathione (0·01 M) in Buffer Solution of pH 7.

Control = suspension in buffer solution (0·04 M) of pH 7.

Test = suspension in buffer solution (0·04 M) of pH 7 and 0·01 M in respect to reduced glutathione.

All suspensions were exposed to air.

Temperature 18°C. [Juice] = 1/100.

Test and control suspensions were inoculated on opposite halves of the same leaves.

Time exposed to air.	5 mins.	1·5 hours.	3 hours.	6·3 hours.	12 hours.
No. of lesions on 8 half-leaves:					
Control	458	172	50	20	0
	n.s.	*	**	**	**
Test	403	435	355	311	223
		n.s.	n.s.	n.s.	*

n.s. = no significant difference.

* = difference is significant at the 0·02 probability level.

** = difference is significant at the 0·01 probability level.

TABLE 5.

Showing the Preservative Effect on the Virus of Tomato Spotted Wilt of Sodium Thioglycollate (0·01 M) in Buffer Solution of pH 7.

Control = suspension in buffer solution (0·04 M) of pH 7.

Test = suspension in buffer solution (0·04 M) of pH 7 and 0·01 M in respect to neutralized (to pH 7) thioglycollic acid.

All suspensions were exposed to air.

Temperature 17°C. [Juice] = 1/100.

Time exposed to air	10 mins.	1·6 hrs.	3·1 hrs.	6·2 hrs.	12·1 hrs.	24 hrs.
No. of lesions on 12 half-leaves:						
Control	179	54	22	10	0	0
	n.s.	**	**	**		
Test	240	227	191	153	18	0
		n.s.	n.s.	n.s.	**	**

n.s. = no significant difference.

* = difference is significant at the 0·02 level of probability.

** = difference is significant at the 0·01 level of probability.

This experiment was performed with two 6 × 6 Latin squares on 36 tobacco plants trimmed to two leaves per plant. Test and control inocula were applied on opposite halves of the same leaves.

Both reduced glutathione and thioglycollate at pH 7 protect the virus against inactivation on exposure to air, but glutathione is a more efficient preservative than thioglycollate.

Ascorbic Acid (Reduced Form).

Ascorbic acid was chosen as a test substance for several reasons. It contains no sulphur, the redox potential of its solutions falls within the range where a protective action on the virus is to be expected if this action is simply connected with the reduction potential, and the tomato plant is particularly rich in the vitamin.

A preliminary test was performed in May, 1934, with a small quantity of the crystalline vitamin prepared from natural sources (B.D.H. product). After five minutes' exposure to air the concentration of active virus in the test suspension (0·01 M in respect to ascorbic acid and buffered at pH 7) was significantly higher than in the corresponding control. The numbers of lesions produced on eight half-leaves were 279 and 135 respectively, However, at the second inoculation performed after a further four hours' exposure to air, neither suspension was infective.

The lower price of the synthetic vitamin made it possible to perform an experiment covering a longer time period, and during June of 1935 two further experiments were carried out—one with ascorbic acid alone and the other with ascorbic acid in the presence of potassium cyanide. The purpose of the cyanide was to inhibit the catalytic oxidation of ascorbic acid by copper and ferric compounds. That such an oxidation of ascorbic acid is catalysed by copper compounds has been shown by Robertson (1934) who also found that it could be arrested by KCN. The results of these experiments are summarized in Tables 6 and 7.

TABLE 6.

Showing the Protective Effect of Ascorbic Acid on the Virus of Tomato Spotted Wilt.

Control = suspension in buffer solution (0·04 M) of pH 7.
Test = suspension in buffer solution (0·04 M) of pH 7 and 0·01 molar in respect to ascorbic acid (neutralized to pH 7).
All suspensions were exposed to air.
Temperature 16°C. [Juice] = 1/100.

Time exposed to air.	5 mins.	1·5 hours.	3 hours.	6 hours.
No. of lesions, 8 half-leaves:				
Control	257	81	19	4
	n.s.	*	*	*
Test	331	223	122	76

(** spans 5 mins. and 1·5 hours.)

n.s. = no significant difference.
* = difference is significant at the 0·02 probability level.
** = difference is significant at the 0·01 probability level.

This experiment was performed with two 4 × 4 Latin squares on 16 tobacco plants trimmed to two leaves per plant. Test and control inocula were applied on opposite halves of the same leaves.

TABLE 7.

A Comparison of the Protective Effects on the Virus of Tomato Spotted Wilt of a Mixture of Ascorbic Acid and KCN, and KCN alone.

Control = suspension in buffer solution (0·01 M) which was also 0·01 M in respect to KCN.

Test suspension in buffer and KCN as for the control but 0·01 M in respect to ascorbic acid as well. Both test and control suspensions were adjusted to pH 7.

All suspensions were exposed to air.

Temperature = 16°C. [Juice] = 1/100.

Time exposed to air.	5 mins.	3 hours.	6·1 hours.	12·5 hours.
No. of lesions, 8 half-leaves:				
Control	285	245	287	430
Test	190	304	288	341
	n.s.	n.s.	n.s.	n.s.
		**		*

n.s. = no significant difference.

* = difference is significant at the 0·02 probability level.

** = difference is significant at the 0·01 probability level.

This experiment was performed with two 4 × 4 Latin squares on 16 tobacco plants from the same potting as those used for the experiment summarized in Table 6, and trimmed to 2 leaves per plant. Test and control inocula were applied on opposite halves of the same leaves.

From Table 6 it is evident that ascorbic acid protected the virus against inactivation on exposure to air. However, the protective effect is not nearly as marked as in the case of cystein or even glutathione, and is more nearly akin to that of thioglycollate. Notwithstanding the protective effect of ascorbic acid, the virus in the test suspension had undergone a progressive inactivation, and at first sight it seemed possible that the protective effect may have been due to an initial reduction of inactivating material present in the juice. However, an examination of the data shows that the inactivation of test and control suspensions proceeded at very different rates. The velocity constants[4] were 0·014 for the control and 0·0048 for the test. The "half life" periods were 50 and 144 minutes respectively. Virus in the control was therefore being inactivated nearly three times as fast as virus in the presence of ascorbic acid. From Table 7 it is evident that when KCN was present as well as ascorbic acid no inactivation at all took place over a period of twelve hours (the duration of the experiment).

In the experiment summarized in Table 7 which covered a period of twelve and a half hours, no virus was inactivated either in the control suspension (KCN) or in the test suspension (KCN + ascorbic acid). In this instance the protective action of KCN alone was more prolonged than in previously reported experiments (Best and Samuel, 1936). There was no significant difference at any inoculation between the test and control inocula.

[4] A study of the reaction velocity of the inactivation processes under various conditions will form the subject matter of a later paper.

Adrenaline.

It was of some interest to determine the effect on the virus of this derivative of catechol. Catechol brings about a practically instantaneous inactivation of the virus in suspensions exposed to air. Adrenaline operates at a more negative redox potential, and as shown by Ball and Clark (1931) its reversibly oxidized form is unstable and has only a transient existence in solution. It seemed likely therefore, that adrenaline would react differently from catechol and that it might even have some protective effect. One experiment was carried out with inocula exposed to air and another with air excluded. The results are summarized in Tables 8 and 9.

TABLE 8.

Showing the Effect of Adrenaline on the Activity of the Virus of Tomato Spotted Wilt.

Control = suspension in buffer solution (0·04 M) of pH 7.
Test = suspension in buffer solution (0·04 M) of pH 7 and 0·01 M in respect to adrenaline.
All suspensions were exposed to air.
Temperature 16°C. [Juice] = 1/100.

Time exposed to air.	6 mins.	1·5 hours.	3 hours.	6 hours.
No. of lesions on 8 half-leaves:				
Control	201	209	123	33
Test	163	196	116	19
	n.s.	n.s.	n.s.	n.s.

n.s. = no significant difference.

This experiment was performed with two 4 × 4 Latin squares on 16 tobacco plants trimmed to two leaves per plant. Test and control inocula were applied on opposite halves of the same leaves.

TABLE 9.

Effect of Adrenaline on the Virus of Tomato Spotted Wilt when Air was Excluded.

Control = suspension in buffer solution (0·04 M) of pH 7.
Test = suspension in buffer solution (0·04 M) of pH 7 and 0·01 in respect to adrenaline.
Both suspensions were maintained in an atmosphere of pure hydrogen.
Temperature 16°C. [Juice] = 1/100.

Time:		12 minutes.	5 hours.
No. of lesions on 12 half-leaves:	Control	205	107
	Test	220	178
		n.s.	†
		**	n.s.

n.s. = no significant difference.
† = difference is significant at the 0·05 probability level.
** = difference is significant at the 0·01 probability level.

This experiment was performed with two groups of six tobacco plants taken at random from a uniform batch of twelve plants. Test and control inocula were applied on opposite halves of the same leaves.

From Table 8 it is seen that there was no significant difference between test and control suspensions at any inoculation. The test suspension was becoming inactive at the same rate as the control.

In the absence of oxygen, however, there was no significant change in the concentration of active virus units after five hours' storage in the presence of adrenaline, whereas the concentration in the control suspension had fallen significantly in this time. This action is similar to that observed with some other reducing agents, and may be attributed to the reduction by adrenaline of the small quantity of oxidant in the juice.

After a few minutes' exposure to air the test suspensions containing adrenaline became brown whereas the test suspension protected against air did not. The reason why the adrenaline did not protect the virus against aerobic inactivation is therefore probably due to an opposing action of the oxidation products of adrenaline (cf. pyrogallate).

Redox Potentials.

The value of the redox potential of a suspension of infective juice in 0·01 M cystein has been reported previously (Best and Samuel, 1936). The values for suspensions of infective juice in 0·01 M solutions of the sodium salts of glutathione, thioglycollic acid and ascorbic acid, and of adrenaline are set out in Table 10. From the literature on the subject it is evident that difficulties have been experienced in obtaining steady potentials with pure solutions of these four substances. In conformity with these experiences the systems containing glutathione and adrenaline gave potentials which became more negative with time, but after five hours what may be regarded as "equilibrium" values were set up.

TABLE 10.

Redox Potentials of Suspensions of the Juice of Tomato Plants Infected with the Virus of Tomato Spotted Wilt, in Solutions of Various Reducing Substances. All Suspensions were Buffered at pH 7 and were 0·01 M in Respect to the Test Substance. For Glutathione the Concentration of Infective Juice was 1 in 50, and for the Rest it was 1 in 100.

A. Oxygen excluded throughout.			B.* After exposure to air.		
Test substance	E_h (volts) at 18°C.		Time exposed	E_h (volts) at 18°C.	
	at 4′.	at equilibrium.	(in hrs.).	at 4′.	at equilibrium.
Glutathione	+ 0·029	— 0·113	12	+ 0·147	+ 0·006
Na thioglycollate	— 0·096	— 0·102	12	+ 0·028	— 0·055
Ascorbic acid (Na salt)	+ 0·062	+ 0·062	6	+ 0·165	+ 0·096
Adrenaline	+ 0·209	+ 0·107	5	+ 0·268	+ 0·123†

* The suspensions were exposed to air for the times specified. Pure nitrogen was then bubbled through the suspensions and the potentials were measured at intervals.

† The exposed adrenaline system was originally a deep red brown but after passing nitrogen for a few hours this colour vanished and the suspension became light green, as when first prepared. All other systems remained bright green even after long exposure to air and still retained their power to reduce phenol indo 2–6 di bromo phenol.

The ascorbic acid system did not vary more than one millivolt over a six-hour period, and the 22-hour (final) reading of the thioglycollate system was only six millivolts more negative than the initial, four-minute, reading. When potentials take several hours to arrive at an equilibrium value it is not always easy to decide whether the drift is due to a slow approach to a true equilibrium in a stable system or to changes in the redox potential of the system. As far as the glutathione and cystein are concerned the work of Dixon and Quastel (1923) and Michaelis (1930) and others has established the fact that a platinum electrode takes some hours to come to equilibrium.

The initial redox potential is in all cases except that of adrenaline, more negative than the critical value for the virus. Even after exposure to air the potentials of the first three on the list are still below the critical value although ascorbic acid is not far below. As expected the potentials are more positive after exposure to air than before. The behaviour of glutathione, thioglycollate and ascorbic acid in respect to the virus fits in quite well with what we would expect from a knowledge of their redox potentials.

The values obtained for adrenaline are difficult to interpret in view of the fact that the potential begins at a value above the critical for the virus but falls fairly rapidly to a value below the critical value. The oxidation products are known to be unstable. Also the suspensions reduced phenol indo 2–6 di bromo phenol, even after exposure for some hours, showing that they still retain the capacity to reduce suitable substrates.

It must be remembered that the redox potential alone does not define the status of a system (in regard to oxidation or reduction). The value of the potential will determine whether a certain reaction will go and in what direction. It is a measure of the intensity factor. However, given a favourable potential, the capacity of a system to oxidize (or reduce) other systems will depend on the amount of active oxidant (or reductant) present.

The rate at which the reaction takes place is another important factor, although this is to some extent connected with the intensity factor.

If we keep these points in mind the behaviour of adrenaline fits in quite well with the known facts.

DISCUSSION.

Before the whole of the differences in lesion numbers recorded in previous sections is ascribed to a preservative effect of the added substance it is necessary to consider other possibilities such as (for example) possible effects of the test substances on the reaction of the host plants.

At inoculation the virus is presumably held to the cell protoplasm by an adsorption process, but it is very probable that some of the test substances enters along with the virus. In the case of a reducing substance this may well provide an environment which is more favourable than usual for multiplication of the virus.

Such action (and the reverse of it in the case of inactivants) must be small, and may in any case be considered ultimately as an action on the virus in the sense that the action is qualitatively similar to the *in vitro* action.

Another probability is that the reducing substance may increase the degree of dispersion of the virus in the suspension. This may result in an increase or a decrease in the number of resultant lesions according to the size of the critical infective unit. The presence of reducing agents does, in fact, result in a greater dispersion of the juice as a whole, but the evidence is against the virus component of the juice being affected in this way. If the above factors and others such as changes in surface tension were operative, we would expect that lesion numbers for the test suspension would be higher than those for the controls by the same proportion in any series of inoculations. However, the ratio $\frac{\text{lesions from test}}{\text{lesions from control}}$ increases progressively (and significantly) with time. Also in a large majority of experiments where the control inoculum is undergoing slow inactivation there is no significant difference between the lesion numbers produced by the test and control inocula at the first inoculation. Further, in those cases where the difference between test and control inocula at the first inoculation is significant, extrapolation of the numbers for the control to zero time shows that the whole of the difference may be accounted for by the greater rate of inactivation of the control suspension.

In those experiments carried out with reducing substances in the absence of air there can be no doubt that these substances are responsible for the preservation, and we may also draw the same conclusion concerning their action in the presence of air.

In a previous paper (Best and Samuel, 1936) the conclusion was drawn that the preservative effect was due to the reducing action as such, provided the redox potential was below $+ 0{\cdot}1$ volt. It was also pointed out that "it so happens that most of the easily accessible reducing agents operating over this potential range which are at the same time not open to objection on account of some other possible action, are sulphur compounds, but since the pyrogallate and hydrogen-platinum systems also protect the virus . . . . the effect may be ascribed to their common action as reducing agents". The sulphur compounds in question were cystein, sodium sulphite, sodium hydrosulphite and sodium sulphide. To these must now be added reduced glutathione and thioglycollate. Pyrogallate, ascorbic acid, H_2 — Pt and KCN are the non-sulphur-containing systems which have a definite protective action. Adrenaline gives only partial protection. The action of KCN may be partly through its action on disulphides to produce sulphydryl compounds, and some evidence for this view was presented in the paper referred to above. However, the instantaneous action of the platinum-hydrogen system and the action of the other non-sulphur-containing systems together with the demonstration of a critical redox potential for inactivation by oxidants makes it evident that the main

effect of the reducing substances is due to their power of acting as reducing agents at potentials more negative than the critical potential for the virus.

However, there are marked differences in the relative efficiencies of the various substances as protective agents in the presence of air. The possibility of some of these differences being due to specific effects cannot be overlooked. Sulphydryl compounds in general and cystein in particular are the most efficient preservatives. Mow much of this is due to the –SH group and how much to other properties remains to be determined. However, it was pointed out previously (Best and Samuel, 1936) that some of these differences may be ascribed to the fact that the oxidation products of the sulphur-containing-compounds are not active oxidants whereas those of pyrogallate are. In the case of ascorbic acid we have another substance which undergoes fairly rapid oxidation in air in solutions at pH 7, under the influence of catalysts. The removal of the protective agent through aerobic oxidation could therefore account for the lower efficiency of ascorbic acid, although the evidence of Table 10 is against this. There is also the possibility that a secondary reaction, in which the oxidised form of ascorbic acid may be involved, may result in virus becoming inactivated, and so opposing reactions would be set up. That such a reaction may inactivate tobacco mosaic virus has been shown by Lojkin (1937).

Finally, there is the possibility of inactivating effects being due to decomposition products. On several occasions hydrogen sulphide has been detected (by odour and the lead acetate test) in samples stored in the presence of cystein.

SUMMARY.

The effect of various reducing systems on the activity of suspensions of the virus of tomato spotted wilt has been tested. Hydrogen in the presence of platinised platinum arrested the normal aerobic inactivation of the virus, and thereafter maintained the activity at a constant level for the duration of the experiment (eight hours).

Suspensions of the virus in the presence of cystein and absence of oxygen have been kept in an active state for 35 days as compared with the normal *in vitro* life of a few hours.

The sodium salts of glutathione, thioglycollic acid and ascorbic acid (all buffered at pH 7) preserved the virus against rapid aerobic inactivation.

Adrenaline had no significant effect on the activity of suspensions exposed to air, but protected the virus against the slow inactivation which takes place in the absence of oxygen.

Redox potentials of the test systems are recorded and discussed in relation to the relative efficiencies of the protective agents.

REFERENCES.

Ball, E. G. and Clark, W. M. (1931): Proc. Nat. Acad. Sci., 17, p. 347.
Best, Rupert J. and Samuel, Geoffrey (1936): Ann. appl. Biol., 23, p. 759.
Best, Rupert J. (1936): Austral. J. exp. Biol., 14, p. 223.
Best, Rupert J. (1937): Austral. J. exp. Biol., 15, p. 65.
Dixon, M. and Quastel, J. H. (1923): J. Chem. Soc., 123, p. 2943.
Lojkin, Mary (1937): Contr. Boyce Thompson Inst., 8, p. 445.
Michaelis, L. (1930): "Oxidation-Reduction Potentials". J. B. Lippincott Co.
Robertson, E. B. (1934): J. Soc. chem. Ind., 53, p. 277.
Samuel, G., Best, R. J. and Bald, J. G. (1935): Ann. appl. Biol., 22, p. 508.

A SEROLOGICAL STUDY OF HAEMOPHILUS INFLUENZAE

2. TWO SEROLOGICALLY ACTIVE PROTEIN FRACTIONS ISOLATED FROM PFEIFFER'S BACILLUS.

by

A. E. PLATT[1]

(From the Department of Bacteriology, University of Adelaide, and the Institute of Medical and Veterinary Science, Adelaide).

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In a previous paper (Platt, 1937) mention was made of an attempt to recover a serologically active fraction common to all strains of *Haemophilus influenzae*. A protein fraction was obtained, but, contrary to expectation, was characterized by a more marked heterogeneity than was the soluble specific substance. In the course of further investigations the existence of a common antigenic factor was demonstrated. To differentiate these two fractions the former has been designated "protein fraction P" and the latter "protein fraction M". Their properties and the method by which they were isolated constitute the subject matter of this paper.

HISTORICAL.

The earliest attempts at classifying strains of *Haemophilus influenzae* revealed a marked heterogeneity of antigenic structure (see Park, Williams and Cooper, 1918; Valentine and Cooper, 1919; Povitzky and Denny, 1921; Coca and Kelly, 1921). The results of these workers weighed against the probability of the existence of a common antigenic factor. Nevertheless, the findings of Maitland and Cameron (1921) and Yabe (1921) suggested that such common antigenic factor existed. The observations of Pittman (1931) supported this view. She found that, under certain conditions, all strains of *Haemophilus influenzae* were agglutinated by an antiserum prepared against any single strain. The results recorded in the author's previous paper (loc. cit.) also supported the view that an antigenic component existed, common to all serological types of Pfeiffer's bacillus.

EXPERIMENTAL WORK.

Preparation of Protein Fraction P.

Organisms grown on solid media only were used. Twenty-four hour Roux-bottle cultures on Fildes' agar were washed off in saline and killed by heating to

[1] The first paper of this series was published in the J. Hyg. Camb., 1937, 37, 98.

55° C. for thirty minutes. The organisms were recovered by centrifugation, washed three times in normal saline and dried *in vacuo* over sulphuric acid. The dried mass was then submitted to prolonged grinding in a bacterial grinder. The resultant very fine powder was extracted with sodium hydroxide solution at pH 7·8 for two hours at 37° C. and overnight in the cold. After removing all insoluble matter by centrifugation at high speed, acetic acid was cautiously added until a precipitate formed. This precipitate was recovered and extracted with distilled water, adjusted to pH 7·6 with sodium hydroxide and the soluble substance re-precipitated with acid. This second precipitate was taken up in normal saline previously adjusted to pH 7·2 with sodium hydroxide. Traces of insoluble matter were removed by prolonged centrifugation. The final solution was perfectly clear and colourless.

A number of "smooth" and "rough" strains were treated in this way. The extracts obtained were examined serologically, and also by injection into animals.

Precipitin Reactions with Fraction P as Antigen.

The constant-antiserum technique (see Dean and Webb, 1926) was used. Several dilutions of antigen were put up against a number of antisera diluted 1 : 5. Each test was put up in duplicate, one being incubated at 37° C. and the other at 55° C. After incubation for four hours the tests were placed overnight in the cold and readings made next morning. Any differences between the readings in the two tests were purely quantitative. The results are given in Table 1.

TABLE 1.

Results of Precipitin Reactions Using Fraction P as Antigen. Reaction Carried Out at 55° C.

	Antisera.								
Antigen	Ly98	MW1	P358	H25	H37	Ly65	H80	L63	Ly110
Ly98	X	—	—	—	—	—	X	—	X
MW1	—	X	—	—	—	—	—	—	—
Ly94	—	—	—	—	—	—	—	—	—
P358	—	—	X	X	—	—	—	—	—
H25	X	—	—	X	—	—	—	—	—
H37	—	—	—	—	X	—	—	tr.	—
Ly10	—	—	—	tr.	—	X	—	—	—
H25R	—	—	—	X	—	—	—	—	—
Ly30	—	—	—	—	X	—	—	—	—

X = Precipitate. — = No precipitate.

Reactions with Antisera Ly94, L23, H53, L95, L35, Ly49, Ly95, H113 uniformly negative.

The high degree of serological specificity shown by this fraction was remarkable. Some of the antisera failed to react with any of the extracts, while others reacted with only a few of them. The lack of agreement between these findings and those obtained with the carbohydrate fractions (Platt, 1937) indicated that

the cross reactions obtained were not due to the presence of traces of specific soluble substance. The results showed equally that this was not the fraction responsible for the close relationship between strains which was demonstrable by agglutination and by precipitin tests using washings from the strains as antigen.

Preparation of Fraction M.

By this time the extremely labile nature of the common antigenic component had been recognized and the need for less drastic methods of extraction was apparent. The solubility of this substance in normal saline had been demonstrated in carrying out the precipitin tests with saline washings from plate cultures. Consequently, twenty-four Roux-bottle cultures on Fildes' agar were washed off in normal saline, adjusted to pH 7·1 with sodium hydroxide and the thick suspension shaken vigorously by hand for five minutes. The organisms were deposited by centrifugation, and the supernatant liquid cautiously made acid in reaction by the addition of dilute acetic acid. At about pH 6·6 a trace of opalescence appeared in the previously water-clear solution. This precipitate was collected by centrifugation and taken up in normal saline adjusted to pH 7·1 with sodium hydroxide. The soluble substance was again precipitated with acid and finally taken up in saline at pH 7·0.

Precipitin Reactions with Fraction M as Antigen.

Extracts from a number of "smooth" and "rough" strains were prepared in this manner and set up in precipitin tests against "smooth" antisera. The results (see Table 2) corresponded closely with those obtained with the crude washings from these strains.

TABLE 2.

Results of Precipitin Reactions Using Fraction M as Antigen. Reaction Carried Out at 55° C.

	Antisera.										
Antigen	Ly98	MW1	Ly94	L23	H53	Ly65	L35	L63	Ly49	H113	Ly110
Ly98	X	—	—	X	X	—	X	X	X	X	X
MW1	X	X	—	X	X	X	X	X	X	X	X
Ly94	—	X	X	X	X	X	X	—	X	—	X
P358	X	X	X	X	X	X	X	X	X	—	X
H25	—	X	X	—	X	X	X	X	—	X	X
H37	X	X	X	X	—	X	X	X	—	X	—
Ly10	—	X	X	X	X	X	X	X	X	X	—
H25R	X	X	X	X	X	X	X	—	X	X	X
Ly30	X	X	X	X	X	X	—	X	X	X	X
H. canis	X	—	—	—	X	X	X	—	X	X	X

X = Precipitate. — = No precipitate.

Reactions with antisera P358, H25, H37, L95, H80, Ly95 uniformly positive.

Physical and Chemical Properties of Fractions.

By freezing out *in vacuo*, fraction P has been obtained as a brownish-white, slightly hygroscopic powder. When dried over a water bath (40° C.) the fraction was obtained as brownish scales almost entirely insoluble in water, saline, or weak alkali. The product obtained by freezing out, however, was readily soluble in saline to which sufficient sodium hydroxide had been added to bring the pH to 7·2. The solution was perfectly clear and colourless. This fraction gave the usual colour reactions for protein. The Molisch reaction was negative.

Fraction M has not yet been obtained as a dried solid. By freezing out, a greyish-white product could be obtained from solutions of this fraction. This amorphous substance was readily soluble in saline rendered neutral (pH = 7·0) with sodium hydroxide, but the solution had no longer possessed the immunological properties of the original.

It is by no means certain that this fraction is a single immunological entity. Solutions of the first precipitate (obtained by adjusting the pH of washings of the organisms to 6·6) gave a weak positive reaction with Molisch's test. After reprecipitation with acid the Molisch reaction was usually negative, but the fraction was less active immunologically as determined by the precipitin test. Although qualitatively the same, the results were quantitatively different; precipitation was slower and the precipitates smaller in amount.

Its extremely labile nature is one of the principal characteristics of this fraction. To measure the degree of its lability the effect of certain physical and chemical agents on its power to react with antisera was noted. It was rendered inactive by exposure to a temperature of 55° C. for four hours. At room temperature it lost its activity in a few days, and in from seven to ten days in the ice chest even though enclosed in a sealed and evacuated tube. It was readily inactivated by acid or alkali, being unable to withstand even the briefest exposure to pH values above 7·2 or below 6·4.

An attempt is being made to obtain larger quantities of this fraction along lines suggested by the recent work of Mudd, Czarnetzky, Pettit and Lackman (1937) on labile antigens.

Toxicity of Fractions.

When injected intraperitoneally into mice protein fraction P produced no ill effects. Protein fraction M, on the other hand, was very toxic, and on intraperitoneal or intravenous injection in mice produced death in about six hours. On post-mortem examination the peritoneum and intestine were found to be moderately congested. The most marked changes were observed in the thoracic cavity. Both the lungs and pleura were markedly congested. Petechial haemorrhages were observed in the parietal and visceral pleura and patches of haemorrhage were present in the lung tissue.

Microscopical examination of a section of the lung showed intense engorgement of the capillaries, transudation of lymph into the alveolar walls and, in many places, rupture of the capillaries with haemorrhage into the alveoli.

DISCUSSION.

In the course of a serological study of *Haemophilus influenzae* eighty-seven nasopharyngeal strains, and four Pittman type strains (Pittman, 1931) of this organism were studied (see Platt, 1937). The examination revealed the extreme antigenic heterogeneity that had been noted by most of the previous workers. The numerous cross reactions obtained, however, indicated that, in addition to an antigen, or antigens, responsible for such marked diversity among the strains, there is another component common to all strains.

The results recorded in this paper suggest that this component is either identical with, or closely related to, the protein fraction M. This fraction was recoverable from all "smooth" and "rough" strains examined for its presence, and the fact that it was readily obtained in solution by simply washing intact bacilli indicated its nearness to the surface of the organism. It will be noted, however, from a perusal of the results recorded in Table 1, that some antisera failed to react with this fraction obtained from certain strains. No satisfactory explanation of this phenomenon can be offered. The results do not indicate the existence of serologically different types within fraction M. It may be that the technique of the precipitin reaction was at fault and that, in tubes where a negative reaction was obtained, the ratio of antigen to antibody lay beyond the limits of a wide range in which a precipitate could form under the conditions of the test. It seems probable also that variations in the anti-M antibody content of the antisera used might be a determining factor.

A similar fraction was obtained from a strain of *Haemophilus canis* (*B. haemoglobinophilus canis*, Friedberger, 1903). This fraction gave precipitates with practically all the antisera against which it was tested. Only one strain of this organism was available, and it was impossible to say, therefore, whether all strains of that species possessed this common antigenic factor.

In view of these findings it seems reasonable to believe that there is an antigenic component common to all strains of *Haemophilus influenzae*. This component is extremely labile. In addition to linking together all strains of influenza bacilli it serves as a connecting link between two species of the genus, viz. *Haemophilus influenzae* and *Haemophilus canis*.

The protein fraction P probably constitutes the bulk of the protein portion of the organism, and the fact that it can be recovered only after gross damage to the organism suggests that it lies deeply within the organism. This fraction showed a high degree of immunological specificity often reacting only with the homologous antiserum.

The evidence available (see also Platt, 1937) suggests that strains of *Haemophilus influenzae* possess three serologically active components, viz. a carbohydrate component situated at the surface of the organism, and which is responsible for dividing the species into a number of serological types; a protein fraction which is situated near the surface and may be closely combined with the carbohydrate fraction, and which is responsible for linking together all serological types within the species and possibly the related species *Haemophilus canis*; a second protein fraction which lies deeply within the organism and, under normal conditions, is rarely exposed at the surface.

SUMMARY.

1. Two protein fractions were recovered from strains of *Haemophilus influenzae* by various methods. These two fractions were designated "M" and "P". Both fractions were serologically active.

2. Fraction M reacted with antisera prepared against most strains, and it appeared to be identical with the antigenic component common to all strains of *Haemophilus influenzae* and at least one strain of *Haemophilus canis*. This fraction is extremely labile.

3. Fraction P is probably situated deeply within the organism. It possesses a high degree of immunological specificity.

REFERENCES.

Coca, A. F. and Kelly, M. F. (1921): J. Immunol., 6, p. 87.
Dean, H. R. and Webb, R. A. (1926): J. Path. and Bact., 29, p. 473.
Friedberger, E. (1903): Zbl. Bakt., 33, p. 401.
Maitland, H. B. and Cameron, G. C. (1921): Brit. J. exp. Path., 2, p. 283.
Mudd, S., Czarnetzky, E. J., Pettit, H. and Lackman, D. (1937): Publ. Hlth. Rep. U.S. Treas. Dept., 52, No. 15.
Park, W. H., Williams, A. W. and Cooper, G. (1918): Proc. Soc. exp. Biol., N.Y., 16, p. 120.
Pittman, M. (1931): J. exp. Med., 53, p. 471.
Platt, A. E. (1937): J. Hyg. Camb., 37, p. 98.
Povitzky, O. R. and Denny, H. T. (1921): J. Immunol., 6, p. 65.
Valentine, E. and Cooper, G. M. (1919): J. Immunol., 4, p. 359.
Yabe, S. (1921): Brit. J. exp. Path., 2, p. 197.

STUDIES ON HAEMOLYTIC STREPTOCOCCI FROM HUMAN SOURCES

2. THE DIFFERENTIATION OF GROUP A (LANCEFIELD) ORGANISMS INTO SEROLOGICAL TYPES (GRIFFITH).

by

G. V. RUDD, COLIN WHITE AND HUGH K. WARD[1]

(From the Department of Bacteriology, University of Sydney).

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Although the classification of Group A haemolytic streptococci into serological types is not so complete as is the similar classification of pneumococci, the types already established by Griffith include the majority of commonly occurring Group A strains.

There are three technical difficulties in the serological typing of Group A organisms, with which every worker in this field has had to contend. They are (1) the production of antisera with a high type-specific agglutinin titre; (2) the absorption of these antisera so that cross-reactions with heterologous types are eliminated; (3) the preparation of suitable suspensions of organisms for slide-agglutination. In this paper we present our own attempts to overcome these difficulties.

We also report the examination of 784 Group A strains isolated in Sydney, the majority of which have been typed.

In the discussion, the distribution of types in Sydney, some aspects of streptococcal epidemiology, and the technical problems of typing are reviewed.

TECHNIQUE.

1. *Source of Material.* We are greatly indebted to Dr. F. Griffith for his Type strains 1–28 with his additional Types "Quinn", "Coggins", and "Roumania". Of these, Types 7, 16, 20, 21 and "Roumania" do not belong to Group A, and we have not included them in this work. In agreement with Pauli and Coburn (1937) we believe that Type 23 is identical with Type 17, and we have therefore eliminated Type 23.

[1] This work was carried out with the assistance of a grant from the National Health and Medical Research Council.

Our own strains have been isolated from (1) throats of normal individuals; (2) throats of patients suffering from Scarlet Fever, tonsillitis, and diphtheria; (3) divers streptococcal lesions.

2. *Preparation of Media.* For the isolation of strains and the examination of colonies, we have employed the special blood agar medium described by Ward and Rudd (1938).

For the preparation of suspensions for agglutination, we have used mainly tryptic broth made according to the technique described by Pauli and Coburn (1937), slightly modified by Ward and Rudd (1938). When one particular type (Type 17) was suspected, we used neopeptone saline as the basis of the medium. This was prepared by dissolving 4·0 gm. of neopeptone in 100·0 c.c. of saline, adjusting the pH to 8·2 and sterilizing in the autoclave.

3. *The Preparation of Suspensions for Slide-Agglutination.* 5·0 c.c. of tryptic broth, to which 0·5 p.c. of ascitic fluid is added, are inoculated from a colony, and incubated for 18 hours at 30° C. The culture is then centrifuged lightly, if the organisms are suspended in the medium, and all but about 0·3 c.c. of the supernatant fluid pipetted off. The organisms are then thoroughly emulsified in this residuum of supernatant fluid with a capillary pipette, and a drop of the suspension placed on a slide and examined with a hand lens. If the suspension is too granular or quickly undergoes auto-agglutination, it cannot be used. In these circumstances, one drop of B.D.H. universal indicator, two drops of Cole and Onslow's pancreatic extract, and enough N/10 NaOH to bring the pH up to about 9·0 are added to the suspension. After a varying time in the incubator at 37° C. the digested suspension will be found suitable for agglutination. Sometimes one hour's digestion will be found sufficient; in other cases it is left overnight in the incubator. We owe this digestion technique to V. D. Allison (unpublished), and although we have used it only towards the end of this study, it seems to be a long step towards the solution of the problem of preparing suitable suspensions from all Group A strains.

Type 17 is commonly associated with Scarlet Fever in Sydney, and invariably yields an unsuitable suspension if grown in tryptic broth. After digestion, the suspension can generally be used for agglutination, but a good suspension can be obtained directly, if this type is grown in 4 p.c. neopeptone saline, to which 5 p.c. ascitic fluid has been added. Type 17 always appears as a mucoid colony on the special blood agar plate.

4. *The Preparation of Agglutinating Antisera.* Each type strain is inoculated into 5·0 c.c. of tryptic broth and incubated for 18 hours at 30° C. The organisms are then centrifuged out, resuspended in 1 p.c. formalin saline solution and the tube placed in the refrigerator for 24 hours. The organisms are again centrifuged out, resuspended in 1·0 c.c. saline solution, and injected intravenously into a rabbit. The injection is repeated after an interval of ten days, and again after an interval of seven days. A fresh vaccine is prepared for each injection.

A little blood is taken from the ear seven days after the third injection, and if the specific titre of the serum is satisfactory, i.e. slide agglutination of the type organism at a dilution of the serum 1/256 or more, the animal is bled out under an anaesthetic, and the serum collected.

5. *The Absorption of Crude Antisera.* The crude antiserum is titrated on the slide against the homologous type organism to obtain the specific titre, and also against a heterologous type organism, usually Type 11, to obtain an idea of its content of heterologous antibody. If the homologous titre is high, and the heterologous titre is very low, no absorption may be necessary; but if the heterologous titre is also fairly high, as is commonly the case, the serum must be absorbed. The absorption is carried out at a dilution of the serum, which is 32 times the homologous titre. If, for example, the homologous titre of a serum is 1/500, and the heterologous titre 1/64, the serum is diluted 1/16 in saline solution, and this diluted serum is absorbed with at least two heterologous types. For this purpose, we used, as a rule, Types 11 and 25.

The diluted serum is absorbed in the following manner: The organisms from a 24-hour culture of Type 11 are centrifuged out, the diluted serum is added to the deposited organisms, which are emulsified in the serum. The tube is placed in the incubator (37° C.) for two hours, after which the organisms are centrifuged out, the serum removed and added to a fresh lot of Type 11 cocci. The tube is incubated at 37° C. for two hours and placed in the refrigerator overnight. Next morning the serum is removed from the Type 11 organisms, and an exactly similar procedure carried out with Type 25. The volume of culture for each absorption is ten times the volume of serum to be absorbed. For example, if a 5·0 c.c. volume of diluted serum is to be absorbed, it is placed in contact with the organisms obtained from 50·0 c.c. of a broth culture of Type 11, and so on.

The serum, after absorption with the two heterologous types, is tested on the slide against suspensions of each of the type organisms. This is a tedious procedure, but it is necessary. If the serum still agglutinates a heterologous type, it is absorbed in the same manner with organisms of that type, and re-tested. When the serum has been proved type-specific, it is heated to 60° C. for one hour to kill any organisms that have not been removed by centrifugation and merthiolate is added to a concentration of 1/50,000.

Should the heterologous titre of the serum be as high as the homologous titre, the serum probably contains little or no homologous antibody, and it is generally useless to attempt to absorb it.

6. *Preparation of Specific Antisera against Certain Group A Haemolytic Streptococci, not Falling into any of Griffith's Types.* As was to be expected, many of our isolated strains were not agglutinated by any of the type antisera. To reduce the number of untyped organisms, we have prepared a few antisera against local strains. Since it might produce confusion in Griffith's classification, if we gave each of these new types a number, we have called each of these new types by

a name, for example, Type "Wade". These new type antisera were prepared in exactly the same way as the antisera against Griffith's types, and were proved to be type-specific before they were used.

7. *Typing of Unknown Strains by Slide Agglutination.* To save time, mixtures of the type antisera are made, each mixture containing four type antisera. Small drops of the suspension of the unknown strain are placed on a clean glass slide, and a loopful of each of the type antisera mixtures is mixed with one of the drops of suspension. One control drop is kept, to which no serum is added. The slide is rocked to and fro, and then the drops are examined for agglutination, using a small hand lens. If agglutination is observed in any of the drops, the particular mixture that was added to that drop is noted, and the suspension is tested in the same manner with each of the type antisera present in that mixture.

Slide agglutination has many advantages, particularly the speed and ease with which it can be carried out. It has the slight disadvantage that the titre of a serum established by one worker may not be the same as that of another worker, even if the same serum and the same suspension are used. This, of course, is due to the impossibility of reproducing the same sized "drop" and the same sized "loop". In the technique we have used, the volume of the drop of suspension is approximately twice the volume of the serum added with the platinum loop. We have attempted to allow for any discrepancy in this proportion of suspension to serum, by using each type antiserum in general 32 times stronger than the end-titre of that antiserum against its homologous type organism. One must bear in mind that the agglutinating effect of an antiserum is reduced four times when present in a mixture containing equal parts of four different type antisera.

8. *Preparation of Group A Carbohydrate.* This was extracted from a Type 12 strain in the manner recently described by A. T. Fuller (1938), and this method of extraction proved to be gratifyingly easy. The titre of this preparation of carbohydrate was 1 : 500,000, when tested with a Group A antiserum by the ring test.

RESULTS.

In all we have attempted to type 784 strains of Group A haemolytic streptococci isolated in Sydney, the great majority of them from patients suffering from Scarlet Fever.

Of these 784 strains, 589 belonging to one or other of Griffith's types, 92 belonged to types not represented in Griffith's classification but established by us, and 103 could not be typed either because the suspension was unsuitable for slide agglutination, or because none of the available type antisera agglutinated the suspensions.

In Table 1 the distribution of the various types is presented. In this table

we have separated the Scarlet Fever strains from the Group A haemolytic streptococci isolated from other sources, because the distribution of Scarlet Fever types is quite different from that of the remainder.

TABLE 1.

Scarlet Fever strains.		Strains isolated from other sources.	
Type 17	242	Type 6	9
Type 1	104	Type "Wade"	8
Type 11	102	Type 28	7
Type 4	63	Type 9	5
Type "Wade"	33	Type 27	5
Type "Gerrard"	21	Type 8	4
Type "Krone"	17	Type 11	4
Type 9	11	Type "Hooper"	4
Type 6	11	Type 4	2
Type 25	5	Type 10	2
Type 28	5	Type 12	2
Type "Hooper"	4	Type 17	1
Type 10	3	Type "Krone"	1
Type 5	1		
Type 27	1		
Total: Typed strains	624	Total: Typed strains	57
Untyped strains	61	Untyped strains	42

(Note: Types "Wade", "Gerrard", "Krone", and "Hooper" are new types established by us).

It will be seen from Table 1 that (a) Types 17, 1, 11 and 4 account for 75 p.c. of the Scarlet Fever strains, while these same types comprise only 12 p.c. of strains isolated from other sources.

(b) Type 17 has a very high incidence (nearly 40 p.c.) among the Scarlet Fever strains. This is largely due to an epidemic in the winter of 1936, when Type 17 was isolated from more than half the cases. No strains were obtained from Scarlet Fever patients in 1937, but in 1938 Type 11 and Type 4 had a higher incidence than Type 17 in Scarlet Fever isolations—see White, Rudd and Ward (1939).

(c) Types 2 and 3, which are very commonly associated with Scarlet Fever in England, were not found at all in Sydney. In both England and Australia, Scarlet Fever remains mild in character.

(d) Several of Griffith's types have not been isolated so far in Sydney, viz. Types 2, 3, 13, 14, 15, 18, 19, 24, "Quinn", "Coggins". From sources other than Sydney, we have identified freshly-isolated strains belonging to Types 2, 13, 15, 19, 24 and "Quinn", so it is unlikely that our technique is at fault.

(e) The untyped strains from Scarlet Fever patients represent only some 10 p.c. of all the strains we attempted to type, while from sources other than Scarlet Fever, we were unable to type some 40 p.c. It is possible that this number will be reduced somewhat when Allison's digestion technique is applied to certain

strains which yielded unsuitable suspensions. Strains found in the routine examination of normal throats have been, in general, the most difficult to identify. We have also failed to type a considerable percentage of strains found in a routine swabbing from the cervix uteri on the third day of the puerperium. These were almost all sporadic infections, and in some cases there was not even a rise in temperature. Apart from Scarlet Fever, we have been most successful with strains isolated from chronically infected tonsils, having identified 14 out of 17 strains from this source.

(f) Three out of our four new type antisera have been useful in this classification, some 11 p.c. of all the strains examined belonging to these new types.

DISCUSSION.

1. *The Relation of Infectivity to Capsulation.*

In a previous paper (Ward and Rudd, 1938) some of the biological characteristics of Group A haemolytic streptococci were described, and it was pointed out that this group of organisms could be divided into capsulated[2] and non-capsulated cocci. In the present study we have attempted to correlate capsulation with the epidemiology of streptococcal infections. Although one may find capsulated and non-capsulated strains within the same serological type, our experience has been that it is exceptional, and that capsulation is associated with some types, while other types are non-capsulated. For example, in our experience in Sydney, Type 17 is always capsulated and Type 11 is always non-capsulated. It should be noted that Types 9 and 11 isolated in Sydney have been non-capsulated, whereas the Griffith's stock strains of these types are capsulated.

A study of the spread of streptococcal infections suggests that the capsulated types are more infectious than the non-capsulated types. Thus, in the winter of 1936, there was a mild epidemic of Scarlet Fever in Sydney, and capsulated organisms (Types 17, 1 and 6) were responsible for 85 p.c. of the infections. In 1938, on the other hand, there was no epidemic, and non-capsulated organisms (Types 4, 9, 11, 12, 25, 27, 28, "Wade", "Gerrard", "Krone" and "Hooper") accounted for 68 p.c. of the infections. More suggestive than these gross figures was the incidence of familial infections. In both periods, the number of familial infections with capsulated organisms was approximately four times as large as the number of familial infections with non-capsulated organisms (White, Rudd, and Ward, 1939).

We have knowledge of only two widespread epidemics of Scarlet Fever in Australia, one in Sydney in 1936 due to Type 17, one in Melbourne in 1938 due to

[2] A correction we have to make is that we inferred in that paper (1938) that streptococci which produced feathery colonies in the serum peptone agar medium were always capsulated. Working with organisms freshly isolated in Sydney instead of Griffith's type strains, we have found that there is no correlation between capsulation and feathery colonies. Capsulated organisms, generally, but not always, grow diffusely in the serum broth tube.

Type 2 (Keogh and Kelsey, 1939). Both Type 17 and Type 2 were shown to be capsulated organisms.

In 1938 we have observed three small outbreaks of Scarlet Fever in institutions; two of these outbreaks were caused by Type 17 (capsulated) and one by Type 1 (capsulated). In the winter of 1937 there was a small epidemic of tonsillitis among the nurses in an obstetric hospital, and at the same time several patients in the hospital contracted puerperal sepsis. The organism responsible for this epidemic was a Type 6 (capsulated) strain. In the last twelve months in the same hospital, puerperal sepsis has been sporadic, and the organisms have been non-capsulated.

Non-capsulated organisms must pass from individual to individual, but we have not observed any obvious spread of infection due to such organisms either in the family, in an institution, or in a community, whereas capsulated organisms on occasion can give rise to an epidemic, never explosive, but one in which the infections are clearly related to one another.

The inference to be drawn from these observations is that capsulated organisms, whether in a lesion or in a carrier, are a greater potential danger than non-capsulated organisms. We are well aware, however, that our experience in streptococcal epidemiology is very limited, as is our evidence on the correlation between capsulation and infectivity. In any case there are, in all probability, additional factors which influence infectivity.

2. *The Technical Problems of Serological Typing.*

In the introduction, mention was made of three difficulties associated with the typing of Group A haemolytic streptococci, and these will now be discussed in the light of our own experience.

(a) *The Production of Antisera.* The method that is usually adopted is to give rabbits a prolonged course of immunization with heat-killed cultures, and even then the method is not uniformly successful. Better results may sometimes be obtained by following up the heat-killed vaccines with injections of living cultures, but there is always the danger of killing the animal. We began the production of antisera on the theory that the reason for the prolonged course of injections found necessary by other workers was the heat-lability of the antigen. It was clear that the type antigen could not be entirely destroyed by heating to 60° C. for one hour, because ultimately such a vaccine does stimulate the production of type-specific antibodies, although failures are apparently not uncommon. On this hypothesis of the heat-lability of the type antigen, we finally adopted the method of killing the culture by formalin at refrigerator temperature and resuspending the dead organisms in saline solution before injection. We took the additional precaution of using a fresh vaccine for each injection. In general, three injections were found to be sufficient. The theory of the heat-lability of the type antigen has been placed on firmer ground by the interesting work of Mudd, Czarnetsky,

Lachman and Pettit (1938). In the beginning of this work we formalinized young (four-hour) cultures for the vaccines, but subsequently we found that nothing was gained by the use of such young cultures.

(b) *The Preparation of Type-specific Antisera from Crude Antisera.* The great majority of antisera cannot be used for typing in their crude state, because they contain too great an amount of heterologous antibodies, which give confusing cross-reactions. With one or two exceptions, the methods adopted for removing heterologous antibody appear to us to be somewhat vague. Naturally, in such a problem, the first question that arises is the nature of these antibodies. Is there one non-type-specific antibody or are there many? If there is only one, the problem of absorption is obviously much simpler. We believe that the first authors to put forward the idea that there was only one such antibody were Mueller and Klise (1933). They suggested that most type antisera contained, in addition to the type antibody, an antibody which reacted with the group antigen, and that this group antigen yielded Lancefield's Group A carbohydrate on treatment with acid. This hypothesis was later taken up by Pauli and Coburn (1937), who were successful in preparing type-specific antisera by the simple procedure of absorbing their crude antisera with Group A carbohydrate. Their technique consisted of choosing an arbitrary dilution of the crude antiserum, an arbitrary concentration of the carbohydrate, mixing the two, incubating and removing the precipitate. We did not have a supply of Group A carbohydrate until very recently, when Fuller (1938) published his method of extracting the carbohydrate from Group A haemolytic streptococci. Our hope that we would be able to prepare type-specific antisera by Pauli and Coburn's simple method was not fulfilled. Indeed, titration of the antisera before and after absorption with the carbohydrate showed that the titre of the non-specific antibody was reduced by no more than 50 p.c. We realize, however, that both our carbohydrate and our antisera were made by methods which differed from those of Pauli and Coburn, so that it is perhaps hardly fair to compare their results with ours.

We cannot answer the question whether there is one heterologous antibody or whether there are several, because in our opinion the data are insufficient. At the same time, our own experience in absorbing a large number of crude antisera makes us doubt whether the group antibody is responsible for all the cross-reactions, although it may well be the most important factor. In the technique we have adopted for preparing type-specific sera, we have compromised by absorbing a calculated dilution of crude antiserum with two type organisms (Types 11 and 25), testing the absorbed serum against all types, and absorbing again with any type that showed a cross-reaction. The merits we can claim for this technique are that it is methodical, and that it has worked well in practice. It has, however, thrown no real light on the antigenic pattern of the Group A haemolytic streptococcus.

(c) *The Preparation of Suspensions Suitable for Slide Agglutination.* As Griffith (1934) has already pointed out, the problem here is to prepare a suspension which is not too granular, and at the same time does not resist agglutination by the type serum. The addition of 5 p.c. ascitic fluid to the culture medium does in most cases increase the stability of the suspension, but we would emphasize Griffith's warning that such suspensions may be inagglutinable by the type serum, and therefore the addition of 5 p.c. ascitic fluid is to be avoided if possible. The age of the culture in the ascitic fluid medium is important, for four-hour cultures of most of the type strains when grown in this medium are inagglutinable, and we have found that inagglutinability is correlated with the presence of a clear halo surrounding the organisms in India ink preparation (Ward and Rudd, 1938). The halo is not present in ink preparations of older cultures, which are then agglutinable.

Allison's technique of digesting granular suspensions may prove to be the answer to this particular problem. We have found it quite satisfactory so far.

3. *The Distribution of Griffith's Serological Types in Sydney.*

It is clear that the distribution of types in Sydney is different from that obtaining in England and America. We have not isolated Types 2 and 3 at all, and only once isolated Type 5 among the first six types. Among the remaining types we have not encountered Types 13, 14, 15, 18, 19, 24, "Quinn", "Coggins". On the other hand, Types 11 and 17 appear to be much more frequently found in Sydney than in England. Of the new types tentatively established by us, Types "Wade", "Gerrard", and "Krone" appear to be fairly common. All these new types grow either as "F" colonies (Ward and Rudd, 1938), or as conical colonies on the special blood plate, and are non-capsulated. The majority of our untyped strains grow in these colonial forms. Mucoid colonies are relatively uncommon in Sydney, apart from Type 17.

Our attempts to classify streptococci from sources other than Scarlet Fever have been somewhat disappointing. Some of our failures are perhaps due to the presence of unknown local types, and it is possible that there are one or two unidentified types which are comparatively common. The clinical infections are sporadic and give no clue as to identity of type.

4. *The Effect of Climate.*

There is a general impression that streptococcal diseases are less common in the tropics than in the temperate climates. Kirschner (1936) has drawn attention to the low incidence of Scarlet Fever and puerperal sepsis in the East Indies. The reason for this must be conjectural until more information is available concerning the types and biological characteristics of Group A haemolytic streptococci that are present in tropical communities.

Sydney has a climate midway between a tropical climate and the more rigorous climate of England and Northern America, and the incidence of streptococcal diseases appears to be lower in Sydney than in those colder countries. For example in the Annual Report of the Department of Public Health of New South Wales, 1934, puerperal sepsis accounted for 15 p.c. of the total maternal mortality, while the corresponding percentage in England was 37·5. Although we have observed a mild Scarlet Fever epidemic and some examples of capsulated cocci obviously spreading from individual to individual, our general experience has been that streptococcal infections in Sydney are sporadic and are caused by non-capsulated cocci. We are at present investigating the seasonal incidence of capsulated and non-capsulated organisms, the results so far suggesting that capsulated cocci are more commonly found in the cooler months of winter and spring. It is conceivable that a cold climate may favour the spread of capsulated cocci, and a warm climate may largely prevent their spread, thus reducing the general incidence of streptococcal infections.

SUMMARY.

784 strains of Group A haemolytic streptococci isolated in Sydney were examined for their serological type—589 belonged to one or other of Griffith's types, 92 belonged to types not represented in Griffith's classifications, and 103 could not be typed.

Griffith's Types 2, 3, 13, 14, 15, 18, 19, 24, "Quinn" and "Coggins" have not been isolated in Sydney up to the present.

Types 17, 11, 1 and 4 accounted for 75 p.c. of the Scarlet Fever strains, while these types accounted for only 12 p.c. of strains from other sources.

Evidence is submitted suggesting that capsulated organisms are apt to be more infectious than non-capsulated organisms.

The technical problems of typing are discussed.

REFERENCES.

Fuller, A. T. (1938): Brit. J. exp. Path., 19, p. 130.
Griffith, F. (1934): J. Hyg., 34, p. 542.
Keogh, E. V. and Kelsey, Helen (1939): Med. J. Austral., I, p. 100.
Kirschner, L. (1936): Geneeskundig Tijdschrift voor Nederlandsch-Indie, Afl. 10 deel 76, p. 596.
Mudd, Czarnetzky, E. J., Lackman, and Pettit, Horace (1938): J. Immunol., 34, p. 117.
Mueller, J. H. and Klise, K. S. (1933): J. infect. Dis., 52, p. 139.
Pauli, Ruth H. and Coburn, Alvin F. (1937): J. exp. Med., 65, p. 595.
Ward, Hugh K. and Rudd, G. V. (1938): Austral. J. exp. Biol., 16, p. 181.
White, Rudd, G. V. and Ward, Hugh K. (1939): Med. J. Austral., 1, p. 96.

THE PROPAGATION OF HERPES, B. AND PSEUDORABIES VIRUSES ON THE CHORIOALLANTOIS

by

F. M. BURNET, DORA LUSH AND A. V. JACKSON[1]

(From the Walter and Eliza Hall Institute, Melbourne).

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As Sabin (1934) has shown, there are distinct similarities in the behaviour of the three viruses, herpes simplex, B. and pseudorabies. All are predominantly but not exclusively neurotropic, all produce intranuclear inclusions, and the particle size of herpes and pseudorabies is the same, around 100 mμ. Herpes virus has been known to be cultivable on the chorioallantois for some years (Saddington, 1932; Dawson, 1933), and recently Mesrobeanu (1938) has described the propagation of pseudorabies virus in this situation. No previous record of the culture of B. virus on the egg membrane has to our knowledge appeared. We have found that all three are readily cultivated by standard methods (Burnet, 1936), producing lesions very similar in macroscopic appearance and in histological structure to those of influenza, ectromelia or myxoma viruses. It seems unnecessary to provide a full description of the characteristics of these viruses on the egg membrane, as it would be almost entirely a repetition of what has been said about other viruses, and in this paper we wish merely to record data bearing mainly on the changes in virulence found after repeated egg passage and on the type of nuclear changes observed in the lesions.

General Character of the Lesions and Changes in Pathogenicity with Egg Passage.

Well established strains of the three viruses were kindly given to us by Dr. E. Weston Hurst in the form of rabbit brain. All three "took" well on the egg membrane immediately, and no difficulty was experienced in maintaining them by egg passage. The herpes strain "H.F." with repeated passage produces somewhat larger foci than in the early stages, and there has been a very steady increase in the amount of virus obtained in a stock virus suspension prepared by grinding one two-day membrane bearing confluent lesions with 5 c.c. broth and centrifuging for ten minutes at 3,000 r.p.m. There has also been an increase in pathogenicity for the embryo which with thirtieth passage virus is usually killed between the third and fifth days. The following series of titres expressed as foci per 0·05 c.c.

[1] Work carried out under grants for research on virus diseases from the Rockefeller Foundation and from the National Health and Medical Research Council.

of stock virus is representative of the results. As a rule one passage has been made per week. Pathogenicity for the rabbit has not noticeably diminished with passage, the virus still provoking keratitis and subsequent encephalitis in the same time as the unadapted virus. It is quantitatively somewhat less pathogenic for mice, an amount capable of producing 40–100 foci on the membrane being required to kill mice on intracerebral inoculation. The strain maintained by mouse passage contains approximately one lethal dose for mice in the amount producing one focus.

TABLE 1.

Increase of Herpes Virus Titre with Continued Egg Passage.

Passage	4	6	8	12	18	29
Titre (in thousands)	1·6	35	68	135	210	900

Another strain of herpes virus ("Tr.") isolated from the human lesion by scarification on the rabbit cornea has also been maintained on the chorioallantois. The pus from the primary keratitis was ground with quartz powder and a gradocol filtrate prepared as the initial egg inoculum. This has shown similar characters, but a lower grade of initial activity; with passage the titre of stock virus suspensions has risen in similar fashion. The strain has after thirty passages lost a good deal of virulence for the rabbit cornea, now producing only a trace of keratitis four to five days after scarification. It was never virulent for mice, killing only a proportion of those inoculated with undiluted virus.

Less work has been done with B. virus, but in most respects it closely resembles herpes. The foci are similar in character but tend to give a wider range of counts from the same diluted inoculum, so that titrations are not so accurate. With a strain having undergone about thirty passages the embryos are killed in four to six days, and frequently show focal lesions in the viscera, virus being present in the embryo in considerable amount. The egg passage virus is still lethal for rabbits by intradermal injection.

Pseudorabies virus grew readily in the eggs from the first passage, giving after two days' incubation thick confluent lesions with the standard virus emulsion and large isolated pocks with higher dilutions. It was decided to passage the virus for a number of generations in the hope that such passage might increase the virulence of the virus for eggs, and at the same time decrease its virulence for laboratory animals such as mice and rabbits. A similar change has been observed in other viruses, in particular the virus of influenza (Burnet, 1936). This hope was only partially realized.

The virulence for eggs did increase somewhat during passage. This increase was shown in three ways: (1) The titre of stock virus prepared from egg membrane with confluent lesions increased about one-hundred-fold over the fifty passages.

(2) Whilst practically all eggs survived inoculation with early passage virus,

it was found that 50 p.c. of eggs inoculated with late passage virus were dead in three days.

(3) It was also noticed that early passage virus, in suitable dilution, produced as a rule large easily countable pocks, whilst eggs inoculated with late passage virus showed many tiny satellite secondary pocks, as well as the larger primary

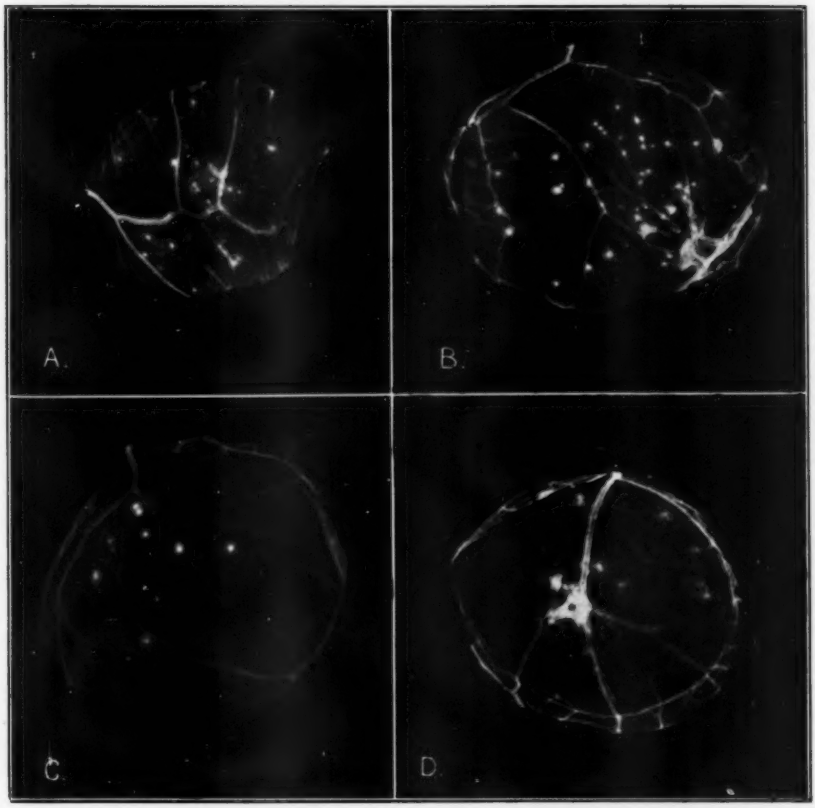

Fig. 1. Egg membrane lesions, natural size. A. Herpes virus. B. B virus. C. Early passage pseudorabies virus. D. Late passage pseudorabies virus, showing primary and secondary foci.

pocks. The secondary pock formation was in fact so marked with the later passages as to make virus titration by pock counting methods very difficult. This can probably also be regarded as indication of increased virulence for the embryo tissues.

Parallel titrations of tenfold dilutions of the virus in eggs and in mice (by intracerebral inoculation) showed that the egg titre and mouse-killing titre were

approximately equal in early passage virus. With late passage virus the titre in mice was a little lower than in eggs and there was a distinctly longer survival time (Table 2).

TABLE 2.

Comparative Pathogenicity for Mice of Early and Late Passage Strains of Pseudorabies Virus.

Dilution of virus.	Fourth passage virus.	Fifty-eighth passage virus.
10^{-6}	6, 11, S, S	S, S, S, S
10^{-5}	5, 9, 9, S	7, S, S, S
10^{-4}	4, 4, 5, 7	3, 7, 7, 9
10^{-3}	3, 3, 3, 4	4, 5, 6, 6
10^{-2}	3, 3, 3, 4	4, 4, 4, 6

The egg titre of both fourth passage and fifty-eighth passage virus was approximately 10^5 per 0·05 c.c. Figures indicate survival time of mice inoculated intracerebrally. S = Survived longer than twelve days.

Rabbits also survived about 24 hours longer after subcutaneous inoculation with the late passage virus than with rabbit or second egg passage material (Table 3). It is of interest that the vigorous scratching of the inoculation site which is characteristic of animals inoculated subcutaneously with animal passage "mad itch" was never seen in rabbits, mice or guinea-pigs inoculated with late egg-passage virus.

TABLE 3.

Pathogenicity for Rabbits of Pseudorabies Virus after Egg Membrane Passage.

Source of Virus.	Survival Time.
Rabbit brain	69 +
Second egg passage	67 +
	79 +
Fifty-third egg passage	91 —
	95 —

Rabbits were inoculated subcutaneously with 0·2 c.c. of standard 5 per cent. emulsion.
+ = scratching of inoculation site.
— = no scratching.

Histology of the Focal Lesions on the Chorioallantois.

The focal lesions produced on the chorioallantois by herpes and B. viruses are macroscopically and histologically similar. There is a certain amount of variation in size, clearness of the margin and degree of inflammatory response from one membrane to another, but there is no significant difference between the two viruses. The development of the lesions takes the same general course as those of other viruses, and particularly resembles that of influenza virus (Burnet, 1936), although proliferation is rather more prominent and necrosis and leucocytic infiltration less so.

The lesion commences with a proliferation of ectodermal cells with partial necrosis of the initially infected ones. There is a rapid accumulation of leucocytes which tend to migrate through the central necrotic area and form a superficial cap with the damaged epithelium. The deeper epithelium continues to proliferate, and at two days there is a fairly sharp differentiation between the necrotic cap and the basal and peripheral mass of better-preserved proliferating ectoderm. In the adjacent mesoderm there is the usual accumulation of leucocytes and histiocytic cells with distended capillaries, to the walls of which leucocytes are adherent. The entoderm may also show an increase to three or four cell layers without evidence of specific infection. Older lesions (five days) are well organized masses of superficial degenerate epithelial cells and relatively normal basal ones with a moderate infiltration with broken-down leucocytes. The mesoderm is still highly cellular, but many of the cells are degenerate, particularly the leucocytes and histiocytes, and fibroblasts are more numerous.

Specific nuclear changes can nearly always be seen, but they do not take the classical form shown for example in herpetic infections of the rabbit cornea. In a two- or three-day membrane lesion the affected cells are most readily found in the deeper proliferating epithelial cells, particularly at the margin of the focus. The most usual form of nuclear change is enlargement with margination of the chromatin, often giving the signet ring appearance. The centre of the nucleus is occupied by lightly staining, slightly acidophil material, usually containing a few large vacuoles and sometimes fragments of chromatin. The appearance strongly suggests distension by some intranuclear activity, and in the more necrotic areas almost completely disrupted nuclei can be recognized by a broken ring of chromatin fragments surrounding acidophil material. In other sections, particularly in older lesions, the nuclear lesions are rather different. There is only slight enlargement, but the margination of the chromatin is complete and the whole interior is filled with a uniform dark staining amorphous material. Very similar changes may be seen in some laryngotracheitis lesions, although more commonly this virus produces typical inclusion bodies in enlarged nuclei. In none of the nuclei from herpes and B. virus egg membrane lesions is the central inclusion separated from the nuclear membrane by a clear space.

If eggs inoculated with B. virus are allowed to incubate for five or six days focal lesions develop in the viscera of the embryo. These are most numerous in the liver, and histological sections show them to be composed of necrotic areas without very much cellular infiltration. At the margins of these areas nuclear changes are very evident. The commonest form is a concentration of chromatin on the nuclear membrane, giving in optical section an appearance like a necklace of irregularly-sized beads. The nuclei are somewhat enlarged and appear to be distended with a uniformly staining acidophilic material. In the central regions of a necrotic focus disrupted nuclei showing only a ring of chromatin dots without any visible nuclear membrane can be seen.

Pseudorabies lesions differ only in the greater prominence of necrotic pro-

cesses. The lesion commences as a proliferation of ectodermal cells, the central cells rapidly undergoing necrosis through the whole thickness of the ectodermal layer. Most of the cells in the proliferating area show nuclear changes, the most characteristic appearance being margination of the chromatin to the periphery of the nucleus, with central vacuolation. However, though a careful search was made at all stages of development, no typical acidophil intranuclear inclusions were seen. Associated with the ectodermal changes one finds an intense mesodermal reaction with many eosinophil polymorphs and thrombosis of small blood vessels. Beneath this the entoderm shows marked cellular proliferation.

DISCUSSION.

The chorioallantoic technique can be used as a standard method of virus and antibody titration for all three viruses, and in the case of herpes at least has the important advantage of allowing the study of strains which are avirulent for mice and rabbits. The method is particularly serviceable for the estimation of antibody against herpes and B. viruses and several studies in this field are in progress or in course of publication.

We were hopeful that continued egg passage might result in sufficient reduction in virulence to allow immunization of rabbits with B. and pseudorabies viruses, but although a slight reduction in virulence of the latter virus was produced, the infection was always fatal. Perhaps the most interesting effect of passage is the disappearance of the scratching and biting of the inoculated region, which is the most characteristic symptom of pseudorabies in rabbits.

SUMMARY.

Herpes, B. and pseudorabies viruses are readily propagated on the chorioallantois of the developing chick embryo.

Herpes and B. virus lesions are similar in character, pseudorabies virus lesions are as a rule larger and more prone to give secondary foci.

With continued passage herpes strains develop increasing pathogenicity for the embryo, as shown by rising titre, and become slightly less virulent for animals.

Pseudorabies virus, after 57 passages on the egg membrane, had become significantly less virulent for mice and rabbits.

The histology of the membrane lesions is briefly described. All show nuclear changes which appear to be specific but differ considerably from the typical intranuclear inclusion.

REFERENCES.

Burnet, F. M. (1936): Sp. Rep. Ser. Med. Res. Coun. Lond., No. 220.
Burnet, F. M. (1936): Brit. J. exp. Path., 17, p 282
Dawson, J. R., Jnr. (1933): Amer. J. Path., 9, p. 1.
Mesrobeanu, I. (1938): C.R. Soc. Biol., 127, p. 1183.
Sabin, A. B. (1934): Brit. J. exp. Path., 15, p. 248.
Saddington, R. S. (1932): Proc. Soc. exp. Biol. and Med., 29, p. 1012.

THE RELATIONSHIP OF HERPES AND B. VIRUSES: IMMUNOLOGICAL AND EPIDEMIOLOGICAL CONSIDERATIONS

by

F. M. BURNET, DORA LUSH AND A. V. JACKSON[1]

(From the Walter and Eliza Hall Institute, Melbourne).

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The virus which has been called by Sabin and Wright (1934) B. virus was isolated by them from brain and cord removed post mortem from a laboratory worker who succumbed to an acute ascending myelitis following the bite of an apparently normal rhesus monkey. The virus was also isolated from the same material by Gay and Holden (1933), and is referred to by them as W. virus. No other isolation of the virus appears to have been recorded in the literature. We are indebted to Dr. E. Weston Hurst for providing us with the virus in the form of glycerolated rabbit brain.

The properties of B. virus have been extensively studied by Sabin and co-workers. The rabbit is the most susceptible animal, being the only common laboratory animal which could be infected with the original human material. Infection by any route produces eventually a fatal myelitis and encephalitis; given intradermally an area of inflammation and vesiculation appears, but corneal inoculation does not result in keratitis. Mice are almost completely insusceptible, and guinea-pigs only irregularly develop fatal encephalitis after intracerebral inoculation (Sabin, 1934b). The susceptibility of the monkey is apparently not uniform. Sabin and Wright failed to infect monkeys in America, but later Sabin (1934c) was able to obtain regularly both local skin lesions and (after intracerebral inoculation) severe or fatal encephalitis in rhesus monkeys in England. He ascribed the previous failure to the use of naturally immune monkeys. This may or may not be correct, as in our experience monkeys known to possess no B. antibody may show no symptoms after intracerebral inoculation with B. virus.

The virus produces intranuclear inclusions of the herpes type, and its behaviour has obvious resemblances to a highly neurotropic strain of herpes febrilis virus. Gay and Holden (1933) in fact have regarded it as a somewhat atypical strain of herpes. Sabin in various papers has pointed out significant differences in behaviour such as its pathogenicity for monkeys, production of visceral lesions

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in the rabbit and failure to give keratitis, and he has also made an extensive immunological comparison of the two viruses, with results which may be summarized as follows: Most immune sera were relatively specific, particularly those prepared against B. virus in guinea-pigs and herpes in rabbits. Monkey sera were, however, less specific, and work with them was complicated by the fact that normal monkeys might contain antibodies against one or other virus. Gay and Holden (1933) concluded that in monkey and human sera the two types of antibody were usually together. Active immunity tests by Sabin showed that rabbits immune to herpes usually succumbed to B. unless very small test doses were used. A herpes treated monkey was not rendered resistant to B. Guinea-pigs which had been inoculated with B. virus and developed antibody were slightly resistant to intracerebral inoculation of E.L. herpes virus, but showed normal susceptibility to Bedson's guinea-pig pad strain.

The use of the chorioallantoic method for the growth and titration of B. virus has been described in a recent paper (Burnet, Lush and Jackson, 1939). The method is particularly applicable to estimations of antibody, and in view of the relation known to exist between B. and herpes viruses we considered it worth while to re-study the antigenic relationship between the two viruses, using human and monkey sera as well as those produced in the ordinary laboratory animals.

1. *The Incidence of B. Virus Antibody in Normal Monkeys.*

A considerable number of monkey sera have been tested in this Institute for the presence of antibody against B. virus by the egg membrane technique. At the same time Dr. E. Weston Hurst was testing sera from several species of monkey in Adelaide, using intradermal inoculation of rabbits with serum-virus mixtures as the method for the detection of antibody. He suggested that his results should be published along with our own, and we are very deeply indebted to him for the material.

The tests made in Adelaide were carried out according to the following technique. Equal volumes of serum and of virus suspension prepared from rabbit brain and diluted to contain 10–100 minimum infective doses were mixed and inoculated intradermally, one rabbit being used for each serum. A control rabbit inoculated with virus-normal rabbit serum mixture was used with each batch of tests. The results are combined in Table 1, and the following notes from Dr. Hurst refer to the groups there shown:

A. *Macaca mulatta* (*rhesus*). These are from the original colony at the Adelaide Zoo. They were acquired in 1935 and are now all adult. There have been no deaths in the colony save from fighting, with the exception of one case of Schilder's disease (Hurst and McLennan, 1937).

B. *Macaca mulatta.* A new colony of immature monkeys obtained from Calcutta at the end of 1937.

C. *Macaca irus* (*cynomolgus*). Numbers C.1 and C.2 were adult animals which had been in the Adelaide Zoo for at least four years. C.3–7: Immature animals bought from Singapore at the end of 1937.

D. *Macaca maurus.* Adult monkeys obtained from Macassar at the end of 1937.

The tests made in Melbourne were carried out by the chorioallantoic technique, and the results are expressed in the usual fashion as the percentage of foci produced by virus-undiluted

serum mixtures in terms of the control count without serum. Group E represents sera from rhesus monkeys obtained at various times prior to 1938. Group F is from a consignment of 150 *M. mulatta* obtained from Calcutta in April, 1938. Table 2 shows the results of testing eight sera of Group E and twenty-three of Group F.

A few other monkeys have been tested by the egg membrane method. Two of seven immature *M. irus* (*cynomolgus*) from Singapore had detectable antibody, but in very low titre (3·0 per cent., 4·3 per cent.), while one nearly adult animal of the same species had considerable amount (< 1 per cent.). One *Macaca radiata* (*sinicus*) and one pig-tail monkey (*M. nemestrina*) had no antibody.

TABLE 1.

Antibody Against B. Virus in Sera from Normal Monkeys—Tests by Intradermal Inoculation of Rabbits.

Species and Group.	No.	Result of serum-neutralization test.		Species and Group.	No.	Result of serum-neutralization test.	
M. mulatta Group A	M.1.671	0	C.7	*M. mulatta* Group B	M.10.	P.9	
	M.2.672	P.9			M.11.	P.8	
	,,	0			M.12.	P.12	
	M.3.	0			M.13.	P.8	
	M.4.	0			M.14.	P.8	
	M.5.	0			M.15.	0	C.8
	M.6.	0	C.8		M.16.	P.8	
	M.7.	0			M.17.	P.20	
	M.8.	0			M.18.	P.8	
	M.9.	0			M.19.	P.8	
					M.20.	P.8	
					M.21.	P.8	
M. irus (cynomolgus) Group C	C.1.	P.31	C.7	*M. maurus* Group D	MM.1.	0	
	C.2.	0			MM.2.	P.10	
	C.3.	P.12			MM.3.	P.8	C.8
	C.4.	P.16			MM.4.	0	
	C.5.	P.8	C.8		MM.5.	P.8	
	C.6.	P.8					
	C.7.	P.8					

O = Rabbit inoculated intradermally remained well.
P.8 = Rabbit inoculated intradermally paralyzed on eighth day.
C.8 = Control rabbit with same virus + normal rabbit serum paralyzed on eighth day.

TABLE 2.

B. Antibody in Rhesus Monkeys—Egg Membrane Titrations.

	Number with no antibody. 30–100%.	Number with antibody, and titres obtained.
M. mulatta. Group E	2	5 (1·5%, 0·66%, 0·02%, 0·018%, 0·003%)
M. mulatta. Group F	20	3 (0·15%, 0·03%, 0·023%)

These results are concordant with each other, and with the previously reported incidence of B. antibody in *Macaca mulatta*. Sabin (1934c) found one of thirteen monkeys with antibody; Hurst (1936a) in three separate batches, six of seven, none of five, and none of fourteen, with antibody. In the present report,

omitting Group E, which is not homogeneous, we have one colony, A, in which all possess antibody, one monkey only giving a serum with an irregular action, and two groups in which only an occasional monkey possesses antibody. There are hardly sufficient numbers of *M. irus* or *M. maurus* to make any statement beyond noting the fact that both species may possess antibody.

2. *The Comparative Activity of Normal and Experimental Monkey Sera against Herpes and B. Viruses.*

The eight sera from normal *M. mulatta* monkeys which had been shown to contain B. antibody by egg membrane titrations were also titrated on the chorio-allantois against the egg passage herpes strain "H.F.", with the results shown in Table 3. Fifteen sera containing no significant antibody against B. were equally without significant inactivating effect on herpes.

TABLE 3.

Comparative B. and "H.F." Titres of Sera from Spontaneously Immune Animals.

	Monkey.							
	N.	R.	1.	10.	21.	42.	145.	159.
Against B	0·66	1·5	0·003	0·02	0·018	0·03	0·15	0·023
Against "H.F."	13	0·08	0·01	0·2	0·015	0·03	0·07	0·15

Figures show the percentage of foci produced when undiluted serum is mixed with the virus shown.

It will be seen, both from this table and from Fig. 1, that there is a good deal of variation in the relative strengths of the two antibody activities, but with one exception (Monkey R.) the B. antibody is either of about the same activity as the herpes antibody or more active. There is a clear suggestion from the results that the herpes antibody is merely a group activity of antibody developed as a result of B. virus infection. To determine whether this was correct, several monkeys which preliminary serum tests showed to possess no antibody against either virus were inoculated by various routes with egg passage B. virus.

Two monkeys, M.97 and M.102, were given virus intranasally—one inoculation of 0·5 c.c. stock egg virus into each nostril without any preliminary treatment of the nasal cavity, and another, M.127, received 0·25 c.c. subcutaneously. No symptoms were noted, and a fortnight later they were inoculated intracerebrally along with two control monkeys, M.129 and M.142, with 0·25 c.c. of undiluted stock egg virus. All five survived without symptoms and were bled eighteen days later.

The results (Table 4) showed an unexpected difference from those of monkeys containing "normal" antibody, in that the relative activity of the sera was much higher against herpes virus than against B. In order to make certain that no change in the "neutralizability" of B. egg passage virus had occurred, sera 42 and 159 were retested and gave rather lower values than those in Table 3, 0·002

p.c. and 0·013 p.c. respectively. If anything, the continued passage of B. has increased its susceptibility to antiserum, which is in accord with our experience with other viruses propagated on the egg.

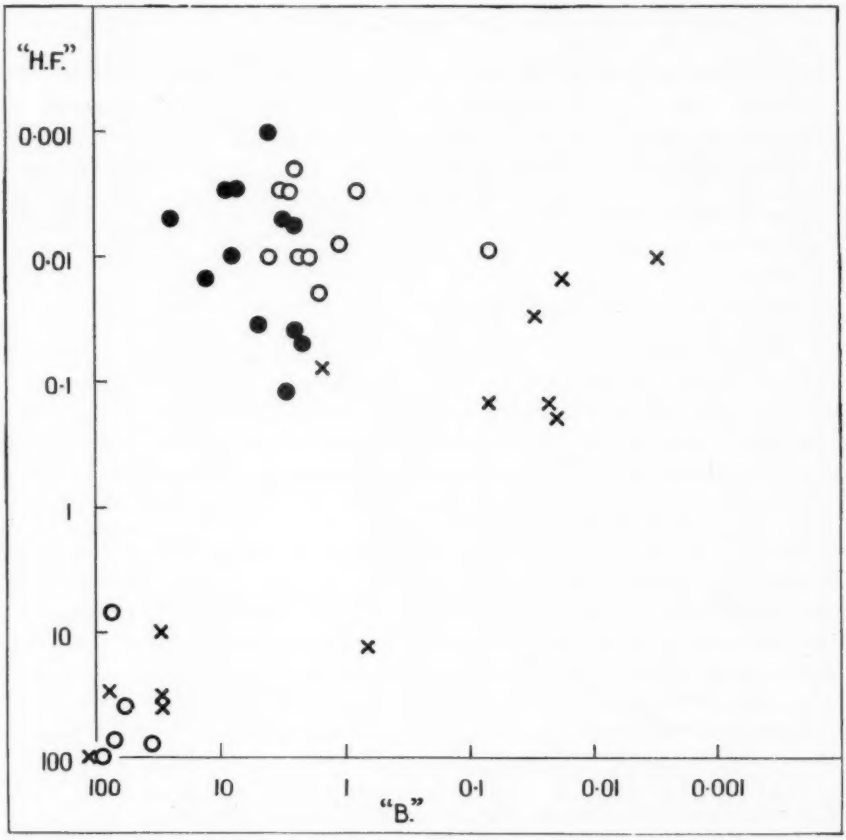

Figure 1. Herpes and B. antibody content of human and rhesus monkey sera. Titres are shown logarithmically in terms of percentage of foci produced on the chorioallantois with undiluted serum.

Open circles	adult human sera.
Black circles	children's sera.
Crosses	M. rhesus sera.

Rhesus monkeys are generally stated to be insusceptible to herpes, but it was rather surprising to find that no antibody at all appeared after intracerebral inoculation of "H.F." (mouse strain), serum-virus mixtures giving the full 100 p.c.

pock count of the controls. This negative result is of value in removing the possibility that herpes antibody resulting from B. inoculation was due to contaminating herpes virus being present in the material used for inoculation.

TABLE 4.

Antibody Content of Sera from Monkeys Infected Experimentally with B. Virus.

Monkey.	First inoculation 12/8/1938.	Sera taken 26/8/1938. B.	"H.F."	Second inoculation 26/8/1938.	Sera taken 13/9/1938. B.	"H.F."
97	B. intranasally	2·2%	0·1%	B. intracerebrally	2·5%	0·085%
102	B. „	2·7%	<0·1%	B. „	1·5%	0·028%
127	B. subcutaneously	12%	0·22%	B. „	5%	0·055%
129				B. „	6·5%	0·075%
142				B. „	4·0%	0·15%

3. *Herpes and B. Antibodies in Human Sera.*

We have titrated a considerable number of human sera from both adults and children for their content of B. antibody, the work being done in association with that on herpes antibody reported in another paper (Burnet and Lush, 1939). Combined results are shown graphically in Fig. 1. This contains only a few of the results from sera without herpes antibody, none of which contained any demonstrable B. antibody. It will be seen that on the whole there is a fairly constant relation between B. and herpes antibody titres, the latter being approximately 100 to 1,000 times more active. One sharp exception is shown, but unfortunately the subject providing the serum was lost trace of before any enquiries into possibly relevant aspects of her history could be made. Children's sera were included, in view of our experience with influenza antibodies (Burnet and Lush, 1938), which showed more specific reactions in children's sera than in adults. The results show a general tendency for B. titres to be lower than those of adults with similar herpes titres, but there is no suggestion of such sharp differences as were found in regard to swine influenza antibodies.

4. *Cross Reactions in Immune Sera from Rabbits and Guinea-pigs.*

Two high titre anti-herpes sera from rabbits were studied in detail. R.158 was immunized with the strain "H.F." by successive intradermal, corneal (moderately severe keratitis) and intracerebral inoculations of living virus. R.161 received corneal and intracerebral inoculations of the strain "Tr.E." recently isolated from recurrent labial herpes. The antibody titres against both herpes strains and B. are shown in Table 5. The ratio between herpes and B. antibody is therefore very similar to that found in human sera.

TABLE 5.

Activity of Herpes Immune Rabbit Sera.

Sera.	Herpes "H.F.E."	Herpes "Tr.E."	B
R.158	0·015%	0·028%	27%
R.161	0·0025%	0·008%	1·5%

We have found, like Sabin, that it is very difficult to immunize rabbits against B. virus, and we have obtained only one rabbit, R.186, which survived intracerebral injection with the virus. This rabbit was inoculated with undiluted B. virus intradermally as a control to two other rabbits which had been given formalized virus. To our surprise it showed no symptoms whatever, either local or general, while one of the immunized animals died. Three weeks later R.186 and the surviving immunized rabbit R.178 were inoculated intracerebrally with undiluted B. virus. R.178 died in six days, R.186 again survived. Unfortunately no serum from R.186 was taken before the first injection, so that we have no indication as to the reason for its quite unusual initial resistance to B. virus. No difficulty was found in rendering guinea-pigs immune by intradermal and subsequent intracerebral inoculation of egg virus, but the serum titres were very poor. The reactions of these four rabbit and guinea-pig sera are shown in Table 6. It will be seen that these anti-B. sera are distinctly more specific than monkey sera, but show more activity against the heterologous virus than do the herpes immune rabbit sera.

TABLE 6.

Activity of B. Immune Sera Against B. and "H.F." Viruses.

Serum.	Antibody titre against B.	Antibody titre against "H.F."
Rabbit 178	3·5%	9·2%
„ 186	0·06%	1·16%
Guinea-pig 153	2·9%	60%
„ 154	2·0%	9%

Serum R.178 taken three weeks after intradermal test injection.
Serum R.186 taken two weeks after last (intracerebral) injection.

5. *Cross Immunity Between Herpes and B. Viruses in the Mouse.*

B. virus is not obviously pathogenic for the mouse, even on intracerebral inoculation. Suitable tests of brain emulsions on the chorioallantois showed that small amounts of virus could be detected up to the sixth day after intracerebral inoculation, but not thereafter. In none of the tests (two mice killed on each of the 1st, 2nd, 3rd, 4th, 6th, 8th and 10th days after inoculation) was any virus detected in the spinal cord. A series of mice were inoculated intracerebrally with

egg membrane emulsion of B. virus, three groups receiving undiluted, 1 : 10 and 1 : 100 stock virus respectively. These were tested with active "H.F." mouse passage virus fourteen days later, with the results shown in Table 7. Unfortunately three non-specific deaths occurred in the series inoculated with B. virus 1 : 10, but the results are sufficient to show that, provided a moderately large immunizing dose is given, there is a substantial immunity developed against herpes virus. Even with the mice given 1 : 100 B. virus there is a significant increase in survival time compared with the controls.

TABLE 7.

Immunity to Herpes of Mice Inoculated Intracerebrally with B. Virus.

Immunizing dose of B. virus.	Test dose of herpes virus 1 : 10.	1 : 100.
Undiluted	S, S, S, S	7, S, S
1 : 10	S	5, S, S
1 : 100	6, 8, 9	6, 7, 10
Nil	4, 4, 5	4, 5, 6

Figures show survival times in days. S = survival for fourteen days.

Pooled serum from mice inoculated intracerebrally with B. virus was of very low titre, and at least as active against herpes virus as against the homologous strain, egg membrane titrations giving the figures: B. 20 p.c., "H.F." 12 p.c.

DISCUSSION.

There is no serious discrepancy between our results and those of Sabin (1934a, b, c), although the general tenor is to indicate a somewhat closer relation between the two viruses herpes and B. than Sabin considers likely.

Clearly there are antigenic factors in common, and on the whole the immunological evidence would suggest that B. virus is a more "complete" antigen than herpes virus. Herpes infections in man and rabbits produce relatively specific antibody, having only about one-thousandth the activity against B. which they show against herpes. Active normal monkey sera, which presumably indicate former natural infection with B. virus, show a much higher heterologous activity, and in the experimentally infected animals the titre against herpes virus is much higher than that against the infecting B. virus. Sera from rabbits and guinea-pigs immunized with B. virus are somewhat more specific, but when allowance is made for their low homologous titre they are considerably less specific than the corresponding herpes sera.

The chief interest of the problem centres on the nature of the infection responsible for the antibodies spontaneously present in a considerable proportion of rhesus sera. There can be no doubt that these antibodies are the result of infection

with a virus of the herpes-B. group. The rather curious finding that monkeys experimentally infected with B. virus gave a much higher antibody titre against herpes than against B., while the active "normal" sera had approximately equal titres against both, shows at least that it is not necessary to postulate infection with more than one virus. The difference may depend either on the fact that normally infection occurs very early in the monkey's life, or that strains of B. virus differ in the extent to which they stimulate the appearance of herpes antibodies.

The fact that a single intranasal instillation of B. virus provoked a well marked antibody response in both the monkeys tested without inducing any noticeable symptoms speaks strongly for the view that B. virus is a long-established parasite of the species. High infectiousness and low morbidity are only seen together with micro-organisms which have worked out a *modus vivendi* with the host species over a long period. Sabin (1934c) suggested that B. was in many respects intermediate between herpes and pseudorabies virus, but based this statement only on pathological and immunological data. An examination of the epidemiological behaviour of the three viruses is equally instructive, and strongly suggests that the three viruses spring from a common stock and have become highly specialized for their respective host species, herpes in man, B. in the monkey, pseudorabies in the pig.

The natural history of herpes infection has been greatly clarified by the recent discovery of Dodd, Johnson and Buddingh (1938) that aphthous stomatitis in infants is almost always an herpetic infection. We have confirmed this, and shown in addition that, unlike other manifestations of herpes, this is accompanied by an absence of antibody in the serum, and is therefore presumably the primary infection (Burnet and Lush, 1939). Herpes, then, is a mild infectious disease of infancy, the virus of which thereafter persists indefinitely in the body, producing occasional local recurrences on the lip or elsewhere. In man it is the most benign of infections, but in the rabbit the virus may behave as a highly invasive neurotrope.

The relation of pseudorabies to the pig appears to be closely similar. Shope (1935) found that the disease in the pig was easily transmitted by the intranasal route, or by contact, but that symptoms following such infection were trivial. Fatal infections could be produced only by intracerebral inoculation. In the middle-west of America the infection seems to be as widespread amongst swine as herpes is amongst hospital patients in Melbourne, the criterion in each case being virus inactivation tests on random samples of serum. Undoubtedly there are occasional epizootics of more serious disease due to pseudorabies virus in swine, but in America at least the normal condition is almost symptomless but rapidly spread infection. The behaviour of the virus in laboratory animals is in striking contrast to that in the pig. It is rapidly fatal with most distressing symptoms in nearly all the common laboratory animals, but only by inoculation; contact infection never occurs.

With B. virus in monkeys the data are not so complete, but they are wholly consistent with an epidemiological behaviour similar to that of herpes and pseudorabies viruses in their respective hosts. The incidence of antibody varies from one batch of monkeys to another, but may on occasion be quite high. The frequency of antibody in the monkeys we have examined is much lower than would be expected if infection occurred in the crowded cages in which they are shipped from India. There is therefore a presumption that, as with human herpes, primary infection occurs mainly during infancy. Non-immune half-grown monkeys, however, are easily infected experimentally by the intranasal route and show no symptoms. Again we find the contrast between the wholly insignificant symptoms in the natural host and severe fatal infection by inoculation in other animals such as man and the rabbit. It is not known whether animals whose blood contains antibody still carry the virus. We have tested gasserian ganglion and submaxillary salivary gland for B. virus in two monkeys with natural antibody, with wholly negative results.

Hurst (personal communciation) has made similar experiments with three monkeys whose sera contained B. antibody, two rhesus and one cynomolgus. Pieces of salivary glands (submaxillary and parotid) were made into a 10 p.c. suspension and inoculated intratesticularly in a rabbit. The testis was passaged to a second rabbit after seven days. The original material was stored for four weeks in glycerol-saline and the test of serial passage through two rabbit testes repeated. All experiments were negative.

Apart from these epidemiological resemblances there are many other reasons for including the three viruses in one group, as Sabin (1934a) and Hurst (1936b) have already suggested. All produce intranuclear inclusions of similar type; herpes and pseudorabies have the same particle size (B. has not been examined in this respect). All are predominantly but not strictly neurotropic; they may appear in the blood after peripheral inoculation, but they enter the central nervous system via the peripheral nerves, not like louping ill, equine encephalomyelitis and some other viruses via blood, olfactory mucosa and olfactory bulb. Another characteristic common feature is their liability to kill experimental animals at a time when very little virus is present in the brain.

SUMMARY.

Tables are given showing the proportion of normal monkeys with antibody against B. virus in several groups of *M. mulatta* and in smaller numbers of monkeys of other species.

Normal monkey sera containing B. antibody also inactivate herpes virus, usually to comparable titre.

Inoculation of monkeys with B. virus by various routes results in the appearance of antibody which is considerably more active against herpes virus than

against the homologous virus. There is no antibody response to inoculation with herpes virus.

Normal human sera which contain herpes antibody have a regular but small activity against B. virus. Herpes immune sera from rabbits have a weak activity against B. of the same order.

Mice inoculated intracerebrally with B. virus show no symptoms, but the virus apparently proliferates to some extent and can be demonstrated up to the sixth day. Mice so treated show a moderately high resistance to herpes.

Sera produced by the immunization of laboratory rodents against B. virus are of low titre and show a considerable cross-activity against herpes virus.

The relationship of B. virus to herpes and pseudorabies viruses is discussed from immunological and epidemiological points of view.

ACKNOWLEDGMENT.

We are much indebted to Dr. E. Weston Hurst for the data on B. antibody in monkey sera which have been included in the text.

REFERENCES.

Burnet, F. M. and Lush, D. (1938): Brit. J. exp. Path., 19, p. 17.
Burnet, F. M. and Lush, D. (1939): Lancet (in press).
Burnet, F. M., Lush, D. and Jackson, A. V. (1939): Austral. J. exp. Biol., 17, p. 35.
Dodd, K., Johnson, L. M. and Buddingh, G. J. (1938): J. Pediatrics, 12, p. 95.
Gay, F. P. and Holden, M. (1933): J. infect. Dis., 53, p. 287.
Hurst, E. W. (1936a): J. exp. Med., 63, p. 449.
Hurst, E. W. (1936b): Brain, 59, p. 1.
Hurst, E. W. and McLennan, G. C. (1937): 8th. Austr. Zoo. and Acclim. Soc., 60th Annual Report.
Sabin, A. B. (1934a): Brit. J. exp. Path., 15, p. 248.
Sabin, A. B. (1934b): *Ibid.*, p. 268.
Sabin, A. B. (1934c): *Ibid.*, p. 321.
Sabin, A. B. and Wright, A. M. (1934): J. exp. Med., 59, p. 115.
Shope, R. E. (1935): J. exp. Med., 62, pp. 85, 101.

FURTHER STUDIES ON THE REACTIONS OF COPPER IONS WITH HAEMOGLOBIN, OXYHAEMOGLOBIN, AND METHAEMOGLOBIN

by

WILLIAM ARTHUR RAWLINSON[1]

(From the Walter and Eliza Hall Institute, Melbourne).

(Submitted for publication 14th December, 1938.)

INTRODUCTION.

In an earlier paper (Rawlinson, 1938) oxyhaemoglobin and native globin were found to be precipitated by the ions of copper, zinc and mercury within definite limits of pH. Cupric ions had a further effect on oxyhaemoglobin, parahaematin being formed; the ferrous iron was oxidized and the protein denatured. It was suggested that the denaturation of the protein in oxyhaemoglobin was an oxidation coupled to the oxidation of the iron atom, because native globin was not denatured by cupric ions under similar conditions.

Holden (1936, 1937) showed that the irreversible denaturation of haemoglobin by acid, alcohol or sodium salicylate occurs only in the presence of oxygen. The experiments reported here are concerned with the effect of copper ions on oxyhaemoglobin, reduced haemoglobin and methaemoglobin, and in particular with the question as to whether denaturation occurs in the absence of oxygen. Since this denaturation proved to be an oxidative phenomenon it was necessary to determine whether it occurred before or after the oxidation of the ferrous atom.

METHODS.

Preparation of Oxyhaemoglobin Solutions.

Both rabbit and bovine pigments were used in the study of denaturation. The erythrocytes were washed four times in saline, lysed with water, adjusted to pH 5·7 for precipitation of the stroma and allowed to stand in the refrigerator overnight; the supernatant solution was then removed and finally readjusted to pH 7·0. The oxyhaemoglobin concentration was determined by coagulation with formalin and weighing. For titration experiments 3·05 p.c. oxyhaemoglobin solutions were used.

[1] The work was carried out under a grant from the National Health and Medical Research Council.

Preparation of Methaemoglobin Solutions.

Methaemoglobin was prepared by the method of Coryell, Stitt and Pauling (1937) with a slight modification. According to these authors oxyhaemoglobin in solutions at pH 4·8–5·3 was oxidized completely on standing at room temperature within 48 hours. In checking this method, solutions of 3 p.c. oxyhaemoglobin were adjusted with acetic acid to pH 4·7 and 5·0 respectively and allowed to stand at room temperature. This experiment was repeated at a temperature of 37°C.

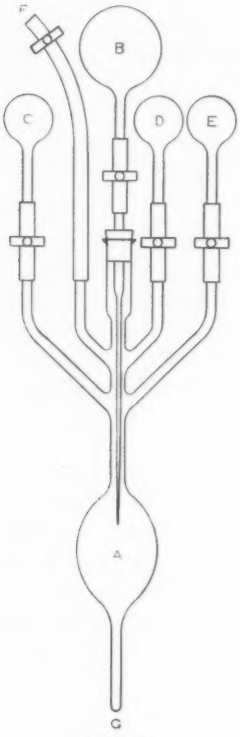

Fig. 1.

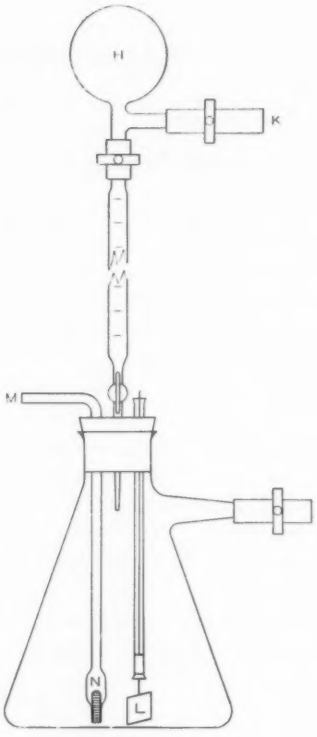

Fig. 2.

At 37°C. oxidation was complete at pH 4·7 and 5·0 within 18 hours; at room temperature oxidation was incomplete in the solution at pH 4·7 after 48 hours, and in that at pH 5·0 even after 96 hours. A negligible amount of precipitation occurred in these solutions during standing. When adjusted to pH 6·7–7·0 there was no further precipitation. No denaturation was detected and the solutions were protected from bacterial decomposition by their low pH. This method was most suitable because oxidizing reagents likely to interfere with subsequent reactions were not required.

For the study of the action of cupric ions in the absence of oxygen, the apparatus illustrated in Fig. 1 was used. By this means copper ions were added to

solutions of the pigments, and any changes which occurred were followed by visual spectroscopy. The main vessel (A) consisted of a bulb, the stem of which had two pairs of side-arms. These side-arms were connected to the bulbs (C), (D) and (E) by means of rubber-tubing provided with screw clips. (C), (D) and (E) contained dilute sodium carbonate, alkaline sodium hydrosulphite and water respectively. Evacuation and equilibration were done at (F). Bulb (B) was larger than the rest and contained the dilute copper sulphate solution; the jet from (B) prevented splashing, and by weighing it together with (B) the amount of copper sulphate added was known. The apparatus was mounted on a board and rotated about GB. A hole in the board at G enabled spectroscopic measurements to be made without dismantling. Six evacuations and equilibrations with hydrogen were carried out by rotating about 30 times per minute for 15 minutes each. The bulbs (B), (C), (D) and (E) were then closed with the screw clips and the main part (A) which contained the reduced haemoglobin was left evacuated in order to facilitate the flow of reagents. The copper sulphate was then added drop by drop and observations made with the Hartridge reversion spectroscope. Finally the solution in (A) was made alkaline and reduced with sodium hydrosulphite and the changes in spectrum noted. The number of equivalents of copper necessary for the oxidation of one equivalent of reduced haemoglobin was determined, the completion of oxidation being observed by the disappearance of the haemoglobin bands. Furthermore denaturation could be detected by observing the changes in the characters of the absorption bands.

Electrometric Method.

The apparatus illustrated in Fig. 2 was devised for the determination of changes in the oxidation-reduction potential accompanying the formation of methaemoglobin from reduced haemoglobin on the addition of copper sulphate, oxygen being excluded as far as possible. The filter flask contained the reduced haemoglobin buffered with sodium acetate-acetic acid solution. Phosphate, borate and citrate buffers were not suitable on account of their reactions with copper sulphate. The electrode (L) used was a spiral of gold wire which appeared to be less readily "poisoned" than bright platinum. Before each determination the electrode was treated with boiling sulphuric acid and rinsed in water. The electrode wire was passed through a gas-tight stopper. A saturated potassium chloride bridge (M) connected the titrating cell to a "saturated" calomel electrode. The end of the bridge which dipped into the titrating flask had an internal ground-glass stopper (N). This allowed electrical contact with minimal polarization but prevented potassium chloride from entering the flask even under reduced pressure. Amplification was obtained by an electrometer valve.

Oxygen was eliminated from each solution before titration in the following manner. The bulb (H) contained the copper sulphate solution and was connected to the burette. The tap of the latter was left open, but the tip was stoppered. Four equilibrations with hydrogen of 15 minutes each were given through the side-arm

(K). The buffered haemoglobin was also equilibrated four times in the filter flask and the burette was then fitted into the stopper. Two more equilibrations were given to the haemoglobin and the flask was left evacuated for the titration. Copper sulphate was added in accurately measured amounts of approximately 1 c.c. and the solution in the flask was kept in rotation until a steady potential was obtained. At the commencement of titration equilibrium was only attained after about 30 minutes, but the time required after each successive addition of copper sulphate was progressively less. The potentials were plotted against the volumes of oxidant and a sudden inflexion in the curve denoted the completion of oxidation. As absolute results were not required the potentials have not been referred to the hydrogen electrode.

The method was checked by using potassium ferricyanide as oxidant, one equivalent being required. Oxidation with ferricyanide was still quantitative in solutions less acidic than in those with copper, although in alkaline solutions even with ferricyanide oxidation was not quantitative. Drabkin and Austin (1936) reported that "at pH values less than 6·0 the pigment solution tended to become turbid". This may have been caused by the presence of excess of ferricyanide. No turbidity was observed in the experiments reported here.

The hydrogen used in all the experiments was freed from oxygen by allowing it to stand over alkaline sodium hydrosulphite for several days.

A Hilger echelon-cell spectrophotometer was used for measuring the ultra-violet absorption of methaemoglobin and a glass-electrode to measure the pH of the solutions.

TABLE 1.

Denaturation by Cupric Ions.

Pigment.	Ratio of Cupric ions to Pigment (Equivalents).	Denaturation.
Haemoglobin	1	None
	2	None
	4	None
	6	Slight
Oxyhaemoglobin	1	Slight
	2	Definite
	3	More Definite
Methaemoglobin	4	None
	10	Slight

RESULTS.

The results of experiments using the apparatus of Fig. 1 are summarized in Table 1. In the case of haemoglobin the formation of methaemoglobin was unattended by denaturation, even when an excess of cupric ions (up to four equivalents) was present. Oxyhaemoglobin was found to be denatured under similar conditions even with two equivalents of cupric ions. These observations therefore suggested that molecular oxygen played an important part in the denaturation.

Ultra-violet spectrophotometric absorption curves showed that denaturation by cupric ions was very much less for methaemoglobin, in the presence of oxygen, than for oxyhaemoglobin. In fact, methaemoglobin appeared to be more stable than reduced haemoglobin to cupric ions. The results of the determination of the number of equivalents of cupric ion necessary for the conversion of haemoglobin and oxyhaemoglobin to methaemoglobin are set out in Table 2. Those obtained by the spectroscopic method showed that with solutions containing no buffer salts and which changed from pH 7 to 6 during titration it was necessary to add 1·4 equivalents of cupric ions for every equivalent of reduced haemoglobin. Oxyhaemoglobin required 2·3 equivalents under similar conditions. When the solutions were buffered to pH 4·7, however, it was found that 1·0 to 1·1 equivalents were sufficient to bring about complete oxidation of both pigments. The oxidation of reduced haemoglobin was more rapid than that of oxyhaemoglobin. The following example is given of a spectroscopic titration.

2·00 c.c. of 3·05 p.c. oxyhaemoglobin buffered to pH 4·7 were titrated with copper sulphate containing 0·001 equivalents per litre. The spectroscopic bands just disappeared after the addition of 3·9 c.c. The number of equivalents of copper sulphate was $0{\cdot}001 \times 3{\cdot}9 \div 1{,}000$. The number of equivalents of haemoglobin was $30{\cdot}5 \times 2{\cdot}0 \div 16{,}700{,}000$. Therefore 1·07 equivalents of copper sulphate oxidized one equivalent of oxyhaemoglobin.

TABLE 2.

Titration Results.

	Method.	pH.	E.
Haemoglobin	Spectroscopic	6–7	1·5
			1·3
			1·6
		4·7	1·0
	Electrometric	5·8	Indefinite
		4·7	1·00
Oxyhaemoglobin	Spectroscopic	6–7	2·2
			2·4
			2·3
		5·6	1·2
		4·7	1·0
			1·0
			1·1
	Electrometric	4·7	Indefinite

E is the number of equivalents of cupric ions causing complete oxidation of one equivalent of pigment.

Electrometric titrations confirmed the spectroscopic results in the case of reduced haemoglobin. In solutions buffered at a pH of 5·8 or over, oxidation was not quantitative, the graph showing no sudden inflexion. At pH 4·7 the potential gave a sudden large increase when 1·00 equivalents of cupric ions had been added to one equivalent of haemoglobin. No information was obtained concerning the

oxidation of oxyhaemoglobin as the potential remained practically unaltered during the titration (see Fig. 3, curve I). The reason for this is discussed below. The following example illustrates the calculation of an electrometric titration.

100 c.c. of 3·05 p.c. reduced haemoglobin containing 40 c.c. of 0·5 M acetate buffer at pH 4·7 were titrated with copper sulphate solution containing 0·020 equivalents per litre and a sudden increase of potential was observed at 9·15 c.c. (see Figure 3, curve II). The number of equivalents of copper sulphate was $0·02 \times 9·15 \div 1,000$. The number of equivalents of haemoglobin was $30·5 \div 16,700$. Therefore 1·00 equivalent of copper sulphate oxidized one equivalent of haemoglobin.

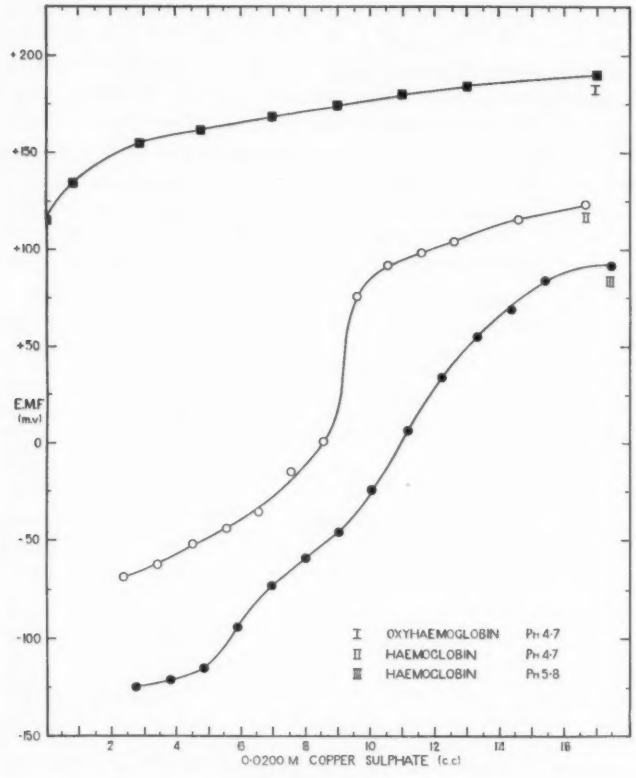

Fig. 3.

DISCUSSION.

The results obtained show that reduced haemoglobin is oxidized to methaemoglobin without being denatured. Oxyhaemoglobin, however, is oxidized more slowly and is more susceptible to denaturation. Methaemoglobin when treated

with cupric ions appears to be more stable than the other two pigments. However, it must be noted that the rapid addition of excess copper sulphate or prolonged standing with an excess causes all three pigments to be denatured. It appears that with concentrations of copper not greatly in excess of that necessary to cause complete oxidation, denaturation of haemoglobin occurs only in the presence of oxygen, and that once the pigment is oxidized to methaemoglobin denaturation is much slower. The denaturation is therefore an oxidation coupled with the oxidation of the prosthetic group.

Oxyhaemoglobin is oxidized less rapidly than reduced haemoglobin because the rate of its oxidation is governed by the rate of its conversion into free haemoglobin. More cupric ions are needed to oxidize both haemoglobin and oxyhaemoglobin in unbuffered solutions apparently on account of the hydrogen-ion concentration, the solution being never more acidic than pH 6·0. Unbuffered oxyhaemoglobin requires 2·3 equivalents of copper, compared with 1·4 for unbuffered reduced haemoglobin. In the presence of oxygen reoxidation of the cuprous ions is a possibility, and the number of equivalents of cupric ions necessary to complete oxidation would then be less than in the case of reduced haemoglobin. When the solutions are buffered to pH 4·7, however, both pigments are found to require one equivalent of cupric ion so that little oxidation of cuprous ions can occur during titration.

The spectroscopic titration was subject to personal variations, and for this reason the oxidation-reduction potential of the system was studied. Previous workers (Conant and Pappenheimer, 1932; Havemann and Wolff, 1937) on methaemoglobin-haemoglobin potential have reported many difficulties. As the potential developed depends on the ratio of oxidized to reduced haemoglobin it is indefinite until some methaemoglobin is formed. With oxyhaemoglobin the amount of free haemoglobin is small; the initial potential is therefore large, and on completion of oxidation no sudden increase of potential is observed. By cleaning the electrode well before use the method gives satisfactory results; equilibrium although slow at the commencement becomes more rapid during the titration. The number of equivalents of copper sulphate required was found to be 1·00 and this agrees with the theoretical equation: Cupric ion with ferrous ion gives cuprous ion and ferric ion, or cupric ion with haemoglobin gives cuprous ion and methaemoglobin.

The cuprous ion formed was suspected as a denaturant. As cuprous salts undergo auto-oxidation and reduction in aqueous solution it is impossible to study their direct action on the blood pigment. Some of these salts are photo-sensitive, but as denaturation was found to occur both in the light and in the dark with apparently the same ease, this possibility was not considered further.

Finally, denaturation of the pigment is not increased by precipitating it within the pH limits found previously (Rawlinson, 1938). In fact denaturation seems to be slower on precipitation apparently because of the decrease in surface area of the protein.

SUMMARY.

Haemoglobin is oxidized by cupric ions to methaemoglobin without denaturation, provided that excess of cupric ions is avoided. Oxyhaemoglobin is partially denatured before the oxidation is complete. If the solution is at pH 4·7, the reaction follows the theoretical equation $Cu'' + Fe'' = Cu' + Fe'''$.

Methaemoglobin may be precipitated unchanged by cupric ions. An excess of cupric ions denatures haemoglobin, oxyhaemoglobin and methaemoglobin whether the pH is suitable for precipitation or not.

ACKNOWLEDGMENT.

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REFERENCES.

Conant, J. B. (1923): J. biol. Chem., 57, p. 401.
Conant, J. B. and Fieser, L. F. (1924): *Ibid.*, 62, p. 595.
Conant, J. B. and Pappenheimer (1932): *Ibid.*, 98, p. 57.
Coryell, C., Stitt, F. and Pauling, L. (1937): J. Amer. Chem. Soc., 59, p 634
Drabkin, D. L. and Austin, J. H. (1936): J. biol. Chem., 112, p. 67.
Havemann, I. and Wolff, K. (1937): Biochem. Z., 293, p. 399.
Holden, H. F. (1936): Austral. J. exp. Biol., 14, p. 291.
Holden, H. F. (1937): *Ibid.*, 15, p. 43.
Rawlinson, W. A. (1938): *Ibid.*, 16, p. 303.

PHOTODYNAMIC ACTION AND THE LIBERATION OF HISTAMINE

by

C. H. KELLAWAY AND E. R. TRETHEWIE[1]

(From the Walter and Eliza Hall Institute, Melbourne).

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The demonstration that various forms of radiant energy (infra-red, visible and ultra-violet) cause liberation of histamine from the perfused lung of the dog (Kellaway, Holden and Trethewie, 1938) led us to enquire whether light could cause the liberation of histamine in greater amounts from tissues sensitized by photodynamic agents. Whereas the direct effects of light involve molecular rearrangements in the tissues but are not necessarily oxidative, photodynamic action is inhibited in the absence of oxygen (Jodlbauer and Tappeiner, 1905; Hasselbalch, 1909; and many others—see Blum, 1932), and is essentially an oxidative process, probably attended by the formation of peroxides. Furthermore Carter (1928) has shown that certain ring compounds, among others histidine, are oxidized by light in the presence of photodynamic agents. If therefore histamine were set free from the tissues by photodynamic action, it might be destroyed as fast as it was liberated, and lung perfusion experiments might fail to yield direct evidence on this point.

Adler (1919) showed that ultra-violet and visible light in the presence of a sensitizer cause contraction of smooth muscle, and Supniewski (1927) has demonstrated that visible light in the presence of haematoporphyrin causes contraction, loss of rhythm and ultimately failure of reaction in smooth muscle from various animals. It seemed possible that photodynamic effects on smooth muscle might yield indirect evidence of the liberation of histamine or of slow-contracting substance.

In the experiments reported here we have studied the effect of visible light on the lungs of dogs sensitized with haematoporphyrin and protoporphyrin, the action of light on histamine in the presence of protoporphyrin, haematoporphyrin, eosin and methylene blue and finally the effects of these photodynamic agents on the isolated jejunum of the guinea-pig.

METHODS.

The isolated lung was prepared as described by Kellaway, Holden and Trethewie (1938). The light sources were a 60 w. globe, 7 cm. distant, screened with 2·2 cm. of running water which

[1] Junior Research Fellow of the National Health and Medical Research Council. This work was aided by a grant from the National Health and Medical Research Council.

gave a reading of 80 on a Weston foot-candle meter, and in a few experiments a quartz mercury vapour lamp, 30 cm. distant, screened with 1 cm. of Wood's glass. In some experiments extracts were made from the perfused right lung using pieces about 1 mm. thick from the surface. In other experiments pieces were taken from various parts of the lung to give an average sample. Similar extracts were made at the end of the experiment from the irradiated left lung, which was weighed before and after to estimate the degree of oedema. The extracts and perfusates were tested on the isolated jejunum of the guinea-pig. When a photodynamic agent was present, the solutions were protected from light and tested in a darkened room. The unboiled perfusate was also tested for slow contracting substance (Feldberg and Kellaway (1938)).

For irradiation, histamine (1: 2 millions in Tyrode solution) was placed in a cell 1 cm. thick whose walls were permeable to visible and ultra-violet light. Photodynamic substances were added to make the concentrations indicated in the text and air was bubbled through at a steady rate. The light source was a 60 w. lamp at 7 cm. distance. In studying the action of light on lung extracts, a series of average extracts from a single lung was made (1 gm. of lung tissue to 20 c.c. Tyrode solution). The histamine free in solution in each sample was estimated before and after irradiation with and without haematoporphyrin 1: 40,000. The extracts were finally boiled to set free any histamine remaining in the cells and cellular débris (Trethewie (1938)) and again tested. In some of these experiments the light was screened with 2·2 cm. of running water to eliminate infra-red and in others the mercury vapour lamp with a Wood's glass filter was used as a light source.

For testing photodynamic action on the isolated guinea-pig's jejunum, this was suspended in oxygenated Tyrode solution in a bath of 5·0 c.c. The room in which the experiments were performed could be darkened by drawing heavy curtains, and enough light to inspect the record and to measure and administer doses was provided momentarily by a minute electric light from a diagnostic set. The light sources used were two 60 w. gas filled electric lights provided with aluminium shades, which were adjusted so as to illuminate somewhat obliquely the upper surface of the bath.

RESULTS.

(a) *Perfused Lungs of Dogs.*

In some of our experiments the lung was first irradiated and then protoporphyrin or haematoporphyrin was injected and it was irradiated again. In others the effect of the injection of porphyrin alone was observed followed by a second injection and exposure to light. The results of experiments in which a slow perfusion rate was used are set out in Table 1, in which also the effect of exposure to light from a 60 w. lamp for half or one hour can be compared with that of the same irradiation upon other lungs in the presence of haematoporphyrin or protoporphyrin. The output of histamine depends to a large extent on the histamine content of the lung. The injection of haematoporphyrin or of protoporphyrin without irradiation caused no appreciable output of histamine, nor after irradiation and injection was there any increase in the histamine in the perfusate above that caused by similar irradiation alone. The output of histamine was too small to cause any appreciable diminution in the amount estimated in average extracts of the left lung as compared with that in the unirradiated right lung. It seemed worth while to compare the histamine content of extracts of the surfaces of both lungs, and this has been done in eight experiments in Table 2, in which in each case the left lung was irradiated with light from a water-screened 60 w. lamp. The

surface samples have not been corrected for oedema because the small amount of fluid which collected in the lungs was in the region of the hilum and in the dependent parts, and the very thin pieces of lung ground up for the surface extracts were thoroughly freed from excess fluid by gentle squeezing between filter papers before weighing. Irradiation with visible light, in contrast to the effect of ultraviolet (Kellaway, Holden and Trethewie, 1938) does not cause any appreciable loss of histamine from the surface of the lung. On the contrary, in one experiment, after irradiation for two hours a rise was observed which was probably caused by drying of the surface despite lavage with the perfusate. In the presence of protoporphyrin or haematoporphyrin there was a significant fall (from 12 p.c. to 43 p.c.) in the histamine present in the surface.

TABLE 1.

Output of Histamine from Perfused Lungs of Dogs by Actinic Light with and without Protoporphyrin and Haematoporphyrin.

Irradiation and Injections.	Histamine in γ per half-hour. Before.	During.		After: in successive half-hours.						Drainage fluid.	Content of lung in γ per gram.
A	Nil	1·6		3·0	2·0	2·5	2·4	3·4	2·0	2·5	25
A	Nil	0·7		5·2	2·8	3·0	2·0			1·8	38
A*	Nil	2·2	2·6	2·0	1·4	1·4					55
A*	Nil	Nil	2·1	1·8	1·2	3·1					35
A)	Nil	2·5		1·5	1·4						
A + p)	1·4	2·2		1·0	1·2					1·8	43
A + p	Nil	2·5		3·1	2·7	2·7	2·8	3·0		1·5	100
p)	Nil			0·3	1·3	0·9	1·0				
B* + p)	1·0	4·6	3·5	3·5	2·1					0·6	42
B)	0·9	3·7		6·5	2·9	2·4					
B + p)	1·6	5·2		6·5	4·4	3·7				5·4	60
h)	Nil			Nil	Nil						
A + h)	Nil	1·3		2·5	1·9	2·6	2·5	2·3	3·2	4·0	55
A + h′	Nil	Nil		0·9	0·9					2·0	15

Explanatory Footnote.

A = Exposure for half-an-hour to 60 w. gas-filled electric bulb at 6 cm. distance screened by 2·2 cm. of flowing water.

B = Exposure for half-an-hour to light from quartz mercury vapour lamp (K.B.B.) at 30 cm. distance screened by 1 cm. of Wood's glass = u.v. 3750–3300 Å.

*= Exposure for one hour.

p = Injection of 5 mg. of sodium protoporphyrinate into cannula in pulmonary artery.

h = Injection of 5 mg. haematoporphyrin dissolved in water with a few drops of 0·1 N NaOH (pH 8·2) into the cannula in the pulmonary artery.

h′ = 20 mg. haematoporphyrin.

These results suggest that histamine is liberated in the course of tissue injury caused by photodynamic action. Our failure to demonstrate this directly in the experiments of Table 1 by increase in the amount of histamine in the perfusate is readily explained because, as we shall show, in the presence of these and other

photodynamic substances, histamine is destroyed rapidly by the light from a water-screened 60 w. lamp. It was conceivable that the additional histamine liberated by photodynamic action was destroyed in the vessels or in the course of collection as fast as it was liberated. The alternative possibility that histamine might be destroyed within the irradiated cells is unlikely because serum protects against this destruction of histamine *in vitro*.

TABLE 2.

Histamine Content of Lung Surface after Irradiation with and without Injection of Haematoporphyrin or Protoporphyrin.

Time of irradiation.	Haematoporphyrin (h) or Protoporphyrin (p).	Right unirradiated.	Left irradiated.
½ hour	5 mg. p	100	75
1 hour	10 mg. h	42	30
1 hour	20 mg. h	40	35
2 hours	30 mg. h	80	46
2 hours	40 mg. h	47	33
1 hour	nil	28	28
2 hours	nil	33	45
2 hours	nil	33	33

The destruction of histamine during perfusion could be reduced by increasing the rate of flow, which in the experiments of Table 1 was only 1·5 to 2·0 c.c. per minute. The results of four experiments in which the rate of flow was 5·0 to 6·0 c.c. per minute and the lung was irradiated for two hours with a water-screened 60 w. lamp at 7 cm. distance are shown in Table 3. In two of these haematoporphyrin was injected in two equal doses at the beginning of irradiation and at the commencement of the second hour of irradiation. The injections were more evenly distributed throughout the lung than in experiments in which the rate of perfusion was slow. The output after haematoporphyrin was more than twice as great as that caused by irradiation alone.

TABLE 3.

Output of Histamine from Perfused Lungs of Dogs after Irradiation with and without Haematoporphyrin.

Amount of haematoporphyrin injected in mg.	Output of histamine in γ in successive half-hours.						Content of histamine in lung in γ per gram.
	Before.	During 2 hours irradiated.				Drainage fluid.	
Nil	Nil	Less than 2·0*	Less than 2·0*	Less than 2·0*	3·8	4·0	59
Nil	Nil	Nil	trace*	trace*	trace*	0·5	33
30	Nil	Nil	5·0	5·2	8·9	5·0	57
40	Nil	2·8	5·4	5·5	6·8	0·7	47

* At the rate of flow 5–6 c.c. per minute the amount of histamine was too small to be estimated accurately.

In none of these experiments was there any increase in the amount of slow contracting substance in the perfusate after irradiation in the presence of protoporphyrin or haematoporphyrin. A solution of slow-contracting substance prepared by H. F. Holden by digesting a suspension of egg yolk with trypsin and subsequent purification with acetone was irradiated for half-an-hour with 1 : 40,000 haematoporphyrin by means of a 60 w. water-screened lamp at 7 cm. distance. After this treatment there was no quantitative nor qualitative change in its reaction upon the isolated jejunum of the guinea-pig. Since the substance is not destroyed by photodynamic action, its absence from the perfusates in these perfusion experiments must be regarded as signifying that it is not liberated from lung tissue damaged by photodynamic action.

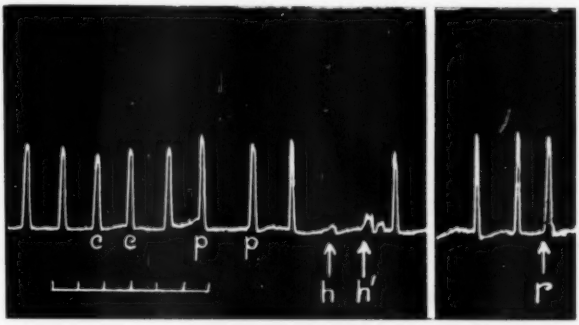

Fig. 1. Responses of isolated jejunum of guinea-pig in the dark to 0·2 c.c. of 1 : 2 millions histamine (unlettered), of the same solution irradiated alone (c), with protoporphyrin (p), with haematoporphyrin (h), to 1·0 c.c. of the histamine solution irradiated with haematoporphyrin (h1), and to irradiated haematoporphyrin with 0·1γ histamine (r). Time in minutes.

Though there was some increase in the amounts of heat-coagulable protein in the perfusate after irradiation in the presence of haematoporphyrin, this was too small to be readily estimated.

(b) *The Destruction of Histamine* in Vitro *by Photodynamic Action.*

Histamine (1 : 2 millions) in a layer 1 cm. thick was irradiated for half-an-hour by a 60 w. lamp 7 cm. distant without loss of activity. The same solution with haematoporphyrin added to make 1 : 40,000 had lost more than 90 p.c. of its activity in half-an-hour. Protoporphyrin was ineffective because it agglutinated in the Tyrode solution in which the histamine was dissolved and haematoporphyrin irradiated alone for the same time did not acquire any added toxicity for the gut which might prevent it from reacting to unirradiated histamine solution. These results are illustrated in Fig. 1.

Serum protects histamine from this destructive action. Histamine 1 : 2 millions made up with 1 : 10 horse serum in Tyrode solution lost 63 p.c. of its activity after irradiation for half-an-hour in the presence of haematoporphyrin 1 : 40,000. When histamine was made up in undiluted horse serum, the observed loss under similar conditions was only 10 p.c.

The effect of ultra-violet light from a quartz mercury vapour lamp at 30 cm. distance screened with 1 cm. of Wood's glass was also tested. Irradiation for half-an-hour with light of this wave length was without effect, nor was there any destruction of histamine when haematoporphyrin was present in a concentration of 1 : 40,000.

Methylene blue was found to be more and eosin rather less active than haematoporphyrin. In Table 4 is shown the percentage destruction caused by these three agents for various times of irradiation of the same strength as in Fig. 1. In estimating these in the case of histamine solution containing eosin and haematoporphyrin increasing doses of these solutions were matched against 0·1γ of unirradiated histamine. Since methylene blue causes contraction of the guinea-pig's jejunum, 0·2 c.c. of the histamine solutions containing it was matched against the same volume of solutions containing 1 : 40,000 methylene blue and varying concentrations of histamine. It will be observed that the photodynamic action of methylene blue is extremely rapid, one-third of the activity of the histamine solution being lost within a minute and 90 p.c. after 10 minutes' irradiation.

TABLE 4.

Percentage Destruction of Histamine (1:2 Millions) by Photodynamic Action.

Time of exposure to light in minutes.	Methylene Blue 1: 40,000.	Eosin 1: 10,000.	Haematoporphyrin 1: 40,000.
1	33		
2	50		
5	80		
10	90	50	33
20	100	71	66
30	—	80	80

It seemed possible that some further help might be obtained in the interpretation of our perfusion experiments upon dog's lungs by examining the effect of irradiation of lung extracts in the presence of haematoporphyrin. For this purpose we used a series of average extracts prepared from the right lung of the third experiment in Table 2 which contained 40γ per gram when all the histamine in the cells was set free into solution by boiling. Our results are set out in Table 5. The variation in the content of free histamine in the four average samples before irradiation was dependent upon the thoroughness with which they were ground, each being prepared separately. The total histamine content obtained after boiling such average samples agrees within 10 p.c. The extract A tested unboiled before and after irradiation with a 60 w. lamp at 7 cm. for half-an-hour was found to contain 20γ of free histamine per gram; after boiling its content was 40γ per

gram lung. The amount of histamine set free by visible light in this experiment was too small to be estimated and there was no appreciable destruction of histamine. In the sample to which haematoporphyrin was added (A + h) the histamine free in solution was 25γ per gram tissue. Following irradiation with the 60 w. lamp 8γ per gram tissue were found and an increase of only 2γ per gram occurred on boiling.

TABLE 5.

The Action of Ultra-Violet and of Visible Light upon Lung Extracts with and without the Addition of Haematoporphyrin.

Content of Extracts in γ Per Gram.

	A	A + h	B	B + h
Before exposure to irradiation	20	25	14	20
After exposure to irradiation	20	8	22	30
After boiling	40	10	36	39

A exposed for half-an-hour to 60 w. lamp at 7 cm. without haematoporphyrin.
A + h exposure as A with haematoporphyrin 1: 40,000.
B exposed for half-an-hour to quartz mercury vapour lamp screened by 1 cm. Wood's glass at 30 cm.
B + h exposure as B with haematoporphyrin 1: 40,000.

In another experiment unboiled extract before and after irradiation for half-an-hour with a water-screened 60 w. lamp gave 17γ and 19γ respectively, and after boiling 38γ per gram was estimated. Here a small amount of histamine was liberated by the action of light but no destruction occurred. A second extract from the same lung similarly irradiated after the addition of haematoporphyrin to make 1: 40,000 gave 25γ before irradiation, 12γ after irradiation and 14γ per gram when finally boiled. The photodynamic action of haematoporphyrin not only caused the destruction of histamine free in solution, but must have been responsible for further liberation of histamine from cells and cellular débris unless destruction of histamine within the cells occurred.

Ultra-violet light filtered through 1 cm. of Wood's glass caused liberation of histamine from cells and cellular débris but no evident destruction of histamine (B). In the presence of haematoporphyrin (B + h) the effect was the same. Haematoporphyrin apparently does not sensitize to ultra-violet light of this wavelength. These observations are in accord with those of Fabre and Simonnet (1926) that the photodynamic action of haematoporphyrin does not occur in the region of 3650Å. Hausmann and Krumpel (1927), on the contrary, found that haematoporphyrin exhibits its maximum absorption in the region of 396μμ to 401μμ with minima on each side of 450–463μμ and 260–263μμ.

(c) *The Isolated Jejunum of the Guinea-pig.*

(1) *Protoporphyrin* dissolved in distilled water in a concentration of 5 mg. per c.c. was added to the bath in doses of 0·2 or 0·1 c.c. (1·0 or 0·5 mg.) and left

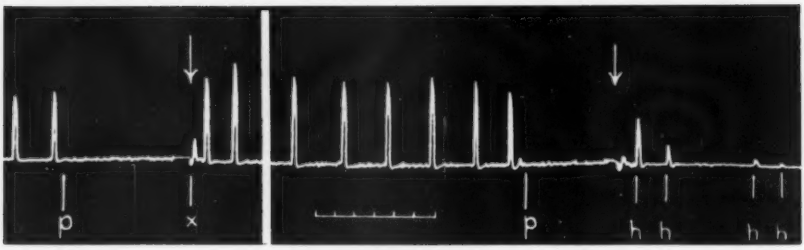

Fig. 2. Responses of isolated jejunum of guinea-pig to repeated doses of 0·1γ histamine. At p, 1 mg. protoporphyrin added to bath. At the arrows, bath washed out and at the second, two 60 w. lamps each 10 cm. distant switched on; x is a spontaneous contraction. The last few contractions to histamine are lettered (h). Time in minutes. The interval between the 1st and 2nd panels is 8 minutes.

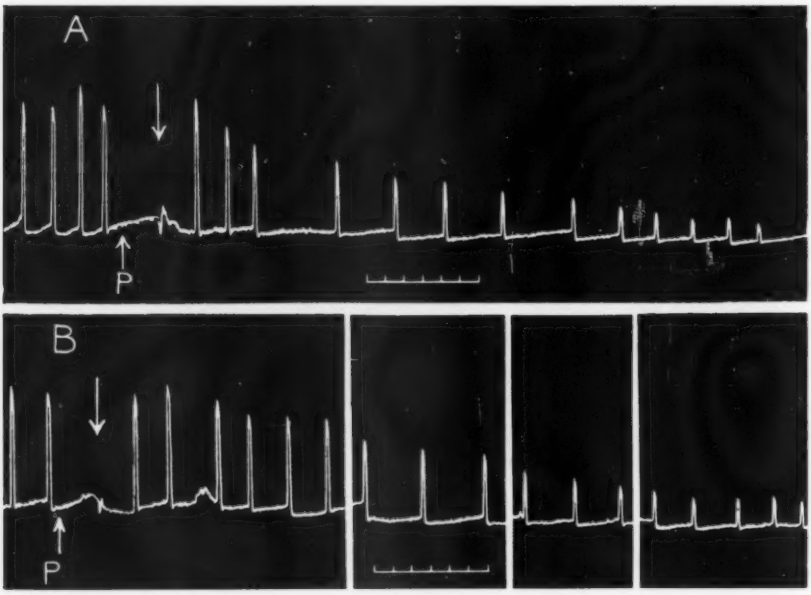

Fig. 3. Records from pieces of jejunum from the same guinea-pig. At P, 0·5 mg. protoporphyrin added to bath. At the arrows (↓) Tyrode solution changed twice and light switched on. The sharp contractions are to repeated doses of 0·1γ histamine. A extends to 40′ after the light was switched on. In B, the end of the second panel is 30′, that of the third 45′ and of the fourth panel 60′ after the light was switched on. Time in minutes. Further details in text.

in contact with the isolated jejunum for two to five minutes in the dark before being thoroughly washed out with two changes of Tyrode solution. By this procedure the tissue was stained, but if kept in the dark appeared to be unaffected and remained just as sensitive to histamine as before. If, however, it was exposed to light it lost its sensitivity to histamine more or less rapidly in accordance with the intensity and distance of the light source. These changes are illustrated in Figs. 2 and 3. In Fig. 2 is seen the absence of effect of 1 mg. of protoporphyrin in contact with the jejunum for five minutes, and tested repeatedly with histamine 0·1γ for twenty-five minutes in the dark before a second dose of protoporphyrin was given in the dark. The gut was then exposed to light for eight-and-a-half minutes, during which it showed a rapid diminution in its response to histamine 0·1γ. Two 60 w. lamps each 10 cm. distant provided the source of light which was shown to be without obvious effect on the unsensitized gut in control experiments.

In Fig. 3 is illustrated, on two pieces of jejunum from the same guinea-pig, the effect of one 60 w. lamp. in A, 20 cm. and in B, 30 cm. distant. In each case 0·5 mg. of protoporphyrin was left in contact with the gut for two minutes, and after washing out the bath twice with Tyrode solution the gut was tested with successive doses of 0·1γ of histamine. In A there is a steady diminution in response in the course of forty minutes and in B a slower falling-off in the course of sixty minutes. Despite the fact that protoporphyrin goes out of solution and agglutinates as soon as it is added to the bath of Tyrode solution it exerts a strong photodynamic effect.

Anticipating that histamine would be liberated in the course of this photodynamic action on the gut we thought it possible that it might cause either (a) a sharp contraction or (b) a slow delayed contraction of the gut accompanying the change from dark to light. We have not observed either of these effects. In the concentrations used protoporphyrin causes an insignificant slow contraction in the dark (Fig. 3). Our observations on the gut gave no support to the view that histamine is liberated by photodynamic action in the isolated jejunum.

(2) *Haematoporphyrin.* This was brought into solution in water at pH 8·2 with 0·1 N NaOH and remained in solution when added to the Tyrode solution in the bath. It was used in doses of 0·2 to 0·5 mg. and left in contact with the jejunum for from one to three minutes. After washing out the bath twice with Tyrode solution, the sensitivity of the gut was tested first in the dark and later in the light after resensitizing the gut by contact with a further dose of haematoporphyrin in the dark. In these doses haematoporphyrin had no effect in the dark on the sensitivity of the jejunum to histamine.

This is illustrated in Fig. 4, which shows the effect of illumination with two 60 w. lamps 10 cm. distant after treatment with haematoporphyrin (0·2 mg.). The responses to repeated doses of histamine (0·1γ) in the dark were not diminished, whereas when the lights were switched on after a second dose of haemato-

porphyrin the gut underwent a continuous loss of excitability, becoming almost completely insensitive to histamine within 30 minutes.

A piece of intestine sensitized with 0·5 mg. of haematoporphyrin left in contact with it for three minutes showed only slightly diminished excitability after two hours' exposure to a 60 w. lamp at 40 cm.

The difficulty in estimating the effect of light of varying strength by the time required to cause failure of response to histamine is that the repeated tests with subsequent changes of the Tyrode solution in the bath wash away the sensitizing substance from the muscle. This difficulty does not arise with protoporphyrin which appears to be fixed more firmly.

In further experiments more than one dose of haematoporphyrin was used, and following the second there was a sharp decline in the response to histamine. This is illustrated in Fig. 5 in which gut was exposed to the light of a 60 w. lamp at 30 cm. A second dose of 0·5 mg. haematoporphyrin was given in the light after 17 minutes and the response to histamine (0·1γ) was at once further reduced.

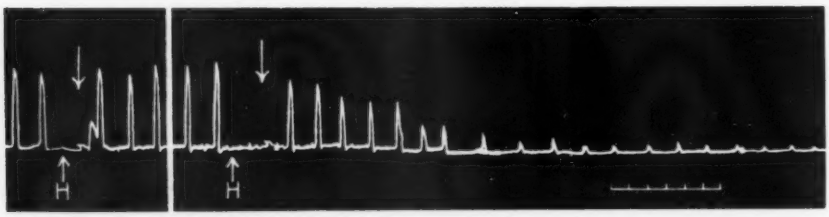

Fig. 4. Responses of isolated jejunum of guinea-pig to repeated doses of 0·1γ histamine. At H, 0·2 mg. haematoporphyrin. At arrows (↓) bath washed out and at that in the second panel lights switched on. Time in minutes. Interval between first and second panels 24 minutes.

There was some recovery of excitability and then a gradual decline, but after 42 minutes the gut still gave a moderate contraction to 0·1γ histamine.

(3) *Methylene Blue* in doses of from 4 to 400γ causes rapid histamine-like contraction of the isolated jejunum of the guinea-pig. When tested in daylight the responses to second and third doses are usually progressively less than to the first (Fig. 6). Since methylene blue is known to sensitize to extremely feeble light, it was possible that this histamine-like contraction was a photodynamic effect. We therefore tested the reaction in complete darkness, the bath being surrounded with an arrangement of black paper and cloth to exclude stray light in the darkened room. Under these conditions the response to a second dose is variable—sometimes more and sometimes less than to the first—but even if the sensitivity is at first depressed it recovers quickly and increases when repeated doses of methylene blue are given in the dark, whereas when the gut is brightly illuminated its sensitivity is invariably and rapidly diminished with repeated doses of methylene blue and no recovery takes place. Figs. 7 and 8 illustrate these effects. In Fig. 7 are seen the responses of a piece of guinea-pig's jejunum to repeated

doses of 50γ methylene blue and 0·1γ histamine (marked h in the tracing). The first part of the record was obtained in complete darkness and at the arrow (↓) after ten doses of methylene blue had been given in the course of 34 minutes in the dark, the gut was irradiated by a single 60 w. lamp at a distance of 10 cm. The interval between the two panels of the figure was 22 minutes. The gut became very insensitive to methylene blue and to histamine during the first six minutes in the light, and showed no recovery during the next 27 minutes. In Fig. 8 two doses

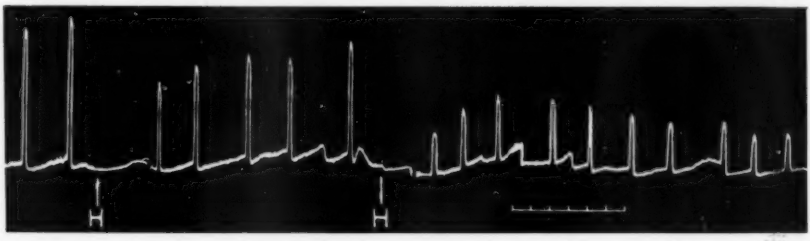

Fig. 5. Responses of isolated jejunum of guinea-pig to repeated doses of histamine (0·1γ) and haematoporphyrin (H) in the light. Time in minutes. Details in text.

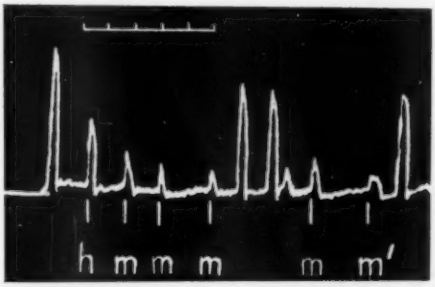

Fig. 6. Responses of isolated jejunum of guinea-pig to histamine and to methylene blue in daylight. The unlettered responses are to 0·1γ of histamine; at h, 0·05γ histamine, at m 10γ and at m′ 4γ methylene blue. Time in minutes.

of histamine 0·1 γ (h) and seven successive doses of methylene blue (50γ) were given during 20 minutes in darkness which was not absolute. The second and third doses of methylene blue gave diminished responses, but the reactivity of the gut then increased and the seventh dose gave a response much greater than that of the first. The response to histamine (0·1γ) was also greatly increased. Light from a 60 w. lamp at 10 cm. caused a rapid decline in the response to methylene blue and to histamine during 20 minutes.

(4) *Eosin.* Both in the light and in the dark eosin in doses of 0·8 to 2·0 mg. (1 : 6,000 to 1 : 2,500) causes slow contraction of the isolated jejunum of the guinea-pig. The contraction can be elicited repeatedly with but little change in its extent. In Fig. 9 are shown five successive responses to 0·8 mg. eosin (e)

(left in contact for two minutes) ; the first three were in the dark and the last two during illumination of the gut with two 60 w. lamps at 10 cm. distance. The figure illustrates the photodynamic action of the dye. During the "dark" period the

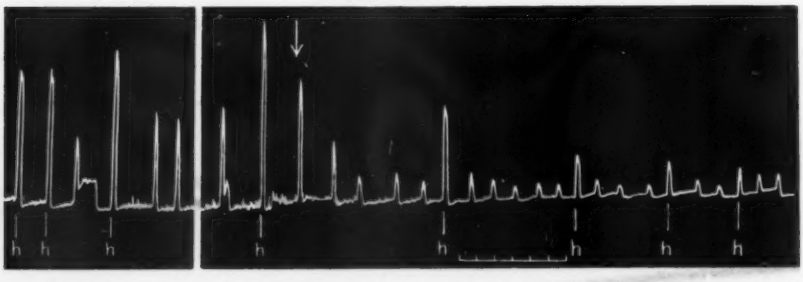

Fig. 7. Responses of isolated jejunum of guinea-pig in absolute darkness and when illuminated to histamine 0·1γ (h) and to methylene blue 50γ (unlettered). Time in minutes. Details in text.

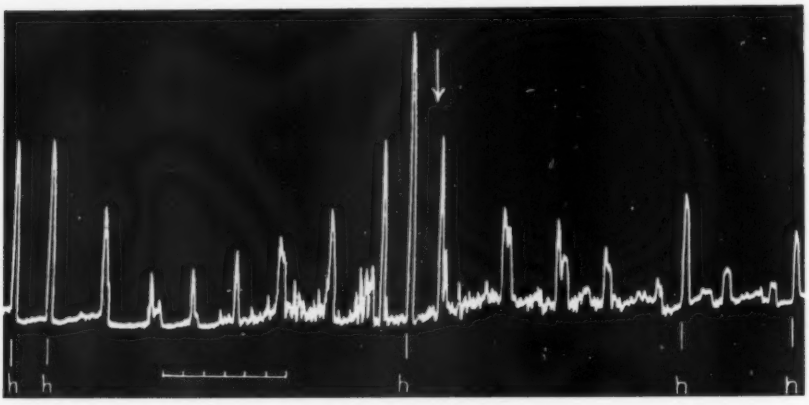

Fig. 8. Responses of isolated jejunum of guinea-pig in dark and light to histamine 0·1γ (h) and to methylene blue, 50γ (unlettered). At arrow light switched on. Time in minutes. Details in text.

responses to 0·1γ histamine steadily increased. Strong illumination, which by itself has no deleterious action on the gut during exposure between half to one hour, caused a diminution of the response to histamine in the gut sensitized with eosin. The first histamine contractions after eosin in the light were followed by a long period of after-contraction. The gut could not be induced to relax by repeatedly changing the Tyrode solution, and the contraction was only cut short by the administration of a further dose of histamine and subsequent washing out of

the bath. Fig. 10 shows the effect of two doses of 1 mg. of eosin (the first left in contact two minutes and the second one minute) on a piece of jejunum from another guinea-pig illuminated as in the experiment of Fig. 9. This illustrates a more usual type of response. The excitability of the gut which is at first increased, progressively decreases during 34 minutes and there are a number of spontaneous contractions (X), only the last of which was attended by emptying of the gut. In

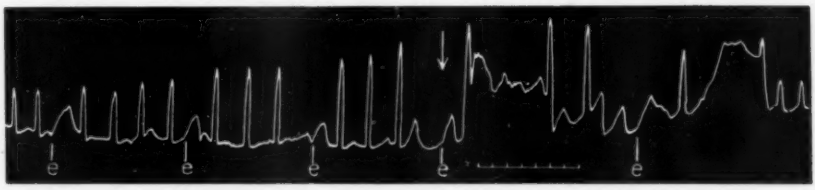

Fig. 9. Responses of isolated jejunum of guinea-pig in the dark and in the light to 0·8 mg. eosin (e) and to histamine 0·1γ (unlettered). Light on at the arrow. Time in minutes. Details in text.

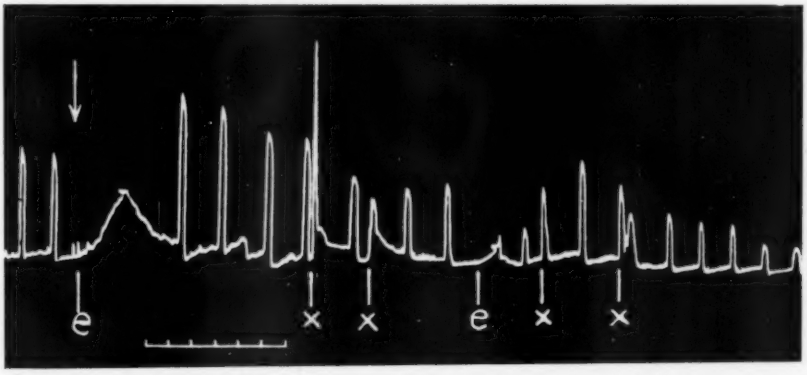

Fig. 10. Responses of isolated jejunum of guinea-pig in strong light to 1·0 mg. eosin (e) and to histamine 0·1γ (unlettered). Light on at the arrow. The contractions marked x were spontaneous. Time in minutes.

another experiment, in which a similar light source was used, a single dose of 2 mg. eosin left in contact with the gut for two minutes reduced the response to histamine 0·1γ to about one-third within half-an-hour. The gut then gave a large emptying contraction; its excitability was further depressed, and did not recover. Such doses of eosin were without injurious effect on the gut during half to one hour in the dark, the response to histamine usually improving, but when given in bright light as in the experiments described above the response to histamine was always much reduced though total loss of excitability was not observed.

These experiments suggest that with the light source used the photodynamic effects of eosin on the guinea-pig's jejunum are not so great as those of the other substances tested; at least the depression of excitability of the muscle is not so dramatic. The repeated tests with histamine tended to remove the dye with which the tissue had been stained and so diminish the effect. Naturally the dye could not be left in contact with the tissue in low concentration since the coloured fluid surrounding the gut would act as a filter for the effective wave-lengths which would not then reach the gut. In all these experiments for the same reason the Tyrode solution containing dye was run off and not allowed to contaminate the fluid in the surrounding water bath.

DISCUSSION.

The severe circulatory symptoms caused by the irradiation of sensitive animals (Hausmann, 1910; Rask and Howell, 1928) must be related to injury of the irradiated skin. They might easily be explained if cellular injury by photodynamic action in this superficial tissue caused the liberation of pharmacologically active substances, though Rask and Howell could obtain no direct evidence in favour of this possibility. The injurious effects of light upon sensitized tissues are not confined to those normally exposed to irradiation and do not depend upon any specific property of surface epithelial cells. Red blood cells are haemolysed (Hasselbalch, 1909); leucocytes are immobilized and lysed (Salvendi, 1906); stasis and accumulation of leucocytes occurs in blood capillaries (Campbell and Hill, 1924); arrhythmias develop in the amphibian (Amsler and Pick, 1918) and mammalian heart (Supniewski, 1927), and skeletal muscle undergoes contraction (Supniewski, 1927; Lippay, 1929, 1930). We were therefore justified in using the isolated dog's lung to investigate the possibility that histamine or other recognizable active substances might be liberated by photodynamic action.

The action of ultra-violet and visible light upon the perfused lungs of dogs sensitized with haematoporphyrin or protoporphyrin though it resulted in a loss of histamine from the irradiated surface of the lung did not, in experiments in which a slow rate of perfusion was used, cause the appearance of significantly more histamine in the perfusate than was produced by the action of ultra-violet or visible light alone. These results did not exclude the possibility that histamine is liberated from the cells by the photodynamic action of light, as well as by its direct action (Kellaway, Holden and Trethewie, 1938), but if this were the case most of the histamine liberated by the sensitizer under the action of light must have been destroyed by photodynamic action in the process of liberation, particularly during irradiation. The experiments of Carter (1928) made it probable that histamine could be so destroyed by oxidation, and we have shown that this is indeed the case.

The photodynamic action of light appears to differ from its direct action upon tissues. Light, particularly ultra-violet, probably produces changes in the chemi-

cal structure of cell constituents which are not necessarily oxidative, whereas photodynamic effects are certainly oxidative whether they take place by the intermediate formation of peroxides or not. Such changes might be expected, if not too severe, to alter the permeability of the cell to its contained histamine, but the chemical structure of histamine is such as to make it peculiarly susceptible to rapid oxidative change by photodynamic action, and we could not expect to obtain easily direct evidence of its liberation by this method.

The fact that the output of histamine from the slowly perfused lung during irradiation with visible light in the presence of haematoporphyrin was not significantly less than that from similarly irradiated control lungs was strongly in favour of liberation of histamine by photodynamic action. During irradiation in the presence of haematoporphyrin much histamine free in solution is destroyed and, unless further histamine were liberated during the process, the output of histamine should be less. The examination of the histamine content of boiled extracts of irradiated surface tissue also suggested that histamine was either destroyed in the cells or had been liberated from them by photodynamic action. Further evidence of an indirect kind for the liberation of histamine by photodynamic action was obtained by the exposure to visible light of suspensions in Tyrode solution of imperfectly ground-up lung tissue. Unless a sensitizer was present visible light set free into solution only small amounts of the histamine in the cells and cellular débris. In the presence of haematoporphyrin there was extremely rapid destruction of histamine and only a trivial amount was left to be set free by boiling. Histamine must therefore have been liberated into the solution by photodynamic action, and subsequently destroyed or must have been destroyed within the cells and cellular débris.

Despite the ease with which it is broken down *in vitro* it is very unlikely that much histamine is destroyed when it is liberated *in vivo* by photodynamic action since it is readily protected by the presence of serum proteins. For the same reason it is unlikely that histamine is destroyed within intact or injured cells by photodynamic action. The accumulation of this indirect evidence for the liberation of histamine by photodynamic action led us to explore the conditions of perfusion under which the least opportunity was afforded for the destruction of histamine. We found that with faster rates of perfusion a significantly greater output of histamine could be demonstrated in the presence of a sensitizing agent.

We have tested the action of visible light in the presence of haematoporphyrin upon the slow-contracting substance which is formed or liberated by other agents which are injurious to the cell (Feldberg and Kellaway, 1938). In the impure form in which we examined it this is not destroyed *in vitro* by the action of light with haematoporphyrin. The photodynamic action on the cells resembles that of the direct action of light, in that this substance does not appear in the perfusate from organs exposed to radiation when a sensitizing substance is present.

The early studies of Adler (1919) showed that some kinds of plain muscle contract under the influence of ultra-violet light, and the later work of Supniewski

(1927) has shown that plain muscle from a number of organs, sensitized with haematoporphyrin, contracts and finally loses its rhythm and becomes insensitive to histamine and to barium salts. Our own experiments with the isolated jejunum of the guinea-pig using several sensitizing agents show clearly the paralysing effects of photodynamic action on this kind of plain muscle, but we obtained no evidence after sensitization of either a rapid or a slow contraction on transition from dark to light. Under the conditions of our experiments if histamine is liberated in the isolated jejunum by photodynamic action it must be destroyed or prevented from exerting its action on the gut by simultaneous changes in the muscle.

CONCLUSIONS.

1. Histamine is liberated from the perfused lungs of dogs by visible light in the presence of haematoporphyrin.

2. It is destroyed *in vitro* by photodynamic action, but this destruction is inhibited by serum.

3. Slow contracting substance is not liberated from the perfused lung of the dog by photodynamic action.

4. We found no evidence of the liberation of histamine by photodynamic action on the isolated jejunum of the guinea-pig.

ACKNOWLEDGMENT.

We wish to acknowledge our indebtedness to Mr. H. F. Holden, who provided us with protoporphyrin and haematoporphyrin for use in these experiments.

REFERENCES.

Adler, L. (1919): Arch. exp. Path. Pharm., 85, p. 152.
Amsler, C. and Pick, E. P. (1918): *Ibid.*, 82, p. 86.
Blum, H. F. (1932): Phys. Rev., 12, p. 23.
Campbell, A. and Hill, L. (1924): Brit. J. exp. Path., 5, p. 317.
Carter, C. W. (1928): Biochem. J., 22, p. 575.
Fabre, R. and Simonnet, H. (1926): Bull. Soc. chim. biol., 8, p. 56.
Fabre, R. and Simonnet, H. (1927): Compt. rend. Acad. Sc., 184, p. 707.
Feldberg, W. and Kellaway, C. H. (1938): J. Physiol., 94, p. 187.
Hasselbalch, K. A. (1909): Biochem. Z., 19, p. 435.
Hausmann, W. (1910): *Ibid.*, 30, p. 276.
Hausmann, W. and Krumpel, O. (1927): *Ibid.*, 186, p. 203.
Jodlbauer, A. and Von Tappeiner, H. (1905): Deutsches Arch. f. klin. Med., 82, p. 520 (cited by Blum).
Kellaway, C. H., Holden, H. F. and Trethewie, E. R. (1938): Austral. J. exp. Biol, 16, p. 331.
Lippay, F. (1929): Pfluger's Arch., 222, p. 616.
Lippay, F. (1930): *Ibid.*, 224, p. 587.
Rask, E. N. and Howell, W. H. (1928): Amer. J. Physiol., 84, p. 363.
Salvendi, H. (1906): Deutsches Arch. f. klin. Med., 87, p. 356 (cited by Blum).
Supniewski, J. V. (1927): J. Physiol., 64, p. 30.
Trethewie, E. R. (1938): Austral. J. exp. Biol., 16, p. 225.

A NOTE ON A CULTURAL TEST FOR GROUP A HAEMOLYTIC STREPTOCOCCI

by

HUGH K. WARD AND G. V. RUDD

(From the Department of Bacteriology, University of Sydney).

(Submitted for publication 20th December, 1938.)

In a recent paper (1938) we described a cultural test for Group A haemolytic streptococci. We made the statement that "there is good correlation between the information afforded by this biological test, and that afforded by Lancefield's serological test". We did not claim that the correlation was perfect. Further work has given very similar results, and we stand by the above statement. At the same time, we wish to draw attention to two pitfalls that were overlooked in our original description of the test.

1. *The Agar Used in the Serum Peptone Agar Medium.* Two lots of agar powder in our own laboratory, one from British Drug Houses, and one from May & Baker, were, and still are, satisfactory when used in the specified concentration of 0·4 p.c. We have, however, tried various lots of powdered agar and shredded agar from other laboratories; none of them was satisfactory in that concentration, but they could be used in a concentration of 0·25 p.c. The peptone agar, when cold, should have the consistency of porridge, and not of a firm gel, as tested by tilting a flask of this medium. In the completed medium, i.e. after the addition of serum and saline, there should be no sense of resistance, when the platinum loop is stirred round during the inoculation of the tube.

Different lots of agar apparently vary considerably in their capacity to gel, and if the medium is too stiff, the colonies will not be characteristic.

Mr. D. Harvey, of the Queen's Memorial Infectious Disease Hospital, Melbourne, has suggested the addition of a small quantity of tryptic broth to the serum peptone agar medium, and we have adopted this suggestion. We have found that in the case of certain Group A strains, the colonies are intermediate between "compact" and "feathery" in our original medium, while in Mr. Harvey's modification of the medium the colonies are definitely "feathery".

Accordingly we now make this medium in the following way: (i) Dissolve 0·25 gm. of shredded agar, 1·0 gm. of neopeptone, 0·85 gm. of NaCl in 100 c.c. of distilled water, adjust pH to 7·6, filter through paper, and sterilize in the autoclave in Flask A. (The contents of this flask, when cold, should have the consistency of porridge, and not of a firm gel. The concentration of agar may have to be altered to obtain the desired consistency). (ii) Into Flask B measure 25 c.c. of tryptic broth, 50 c.c. of saline solution, and 25 c.c. of horse serum (not more than eight weeks old) which has been heated to 56°C. for thirty minutes.

Melt the contents of Flask A and, after cooling to 50–55°C., add to the contents of Flask B, which has been warmed to 50–55°C. Mix and distribute the mixture with a sterile pipette in 3·0 c.c. amounts into sterile 3 in. by ½ in. test tubes. The tubes are stored in the refrigerator and in any case should be cooled down in the refrigerator before use.

2. *Group B Streptococci from Human Sources.* When grown on peptone blood agar, some Group B strains are haemolytic, and these strains cause no confusion, as there is no opalescence when grown in serum broth, and the colonies in the serum peptone agar are compact.

Many Group B strains, however, are not truly haemolytic on the peptone blood agar medium, the colonies being surrounded by a zone of partial haemolysis. The colonies of these strains in serum peptone agar are feathery and have caused confusion. With a little experience, nevertheless, the colony can be distinguished from the Group A "feathery" colony. There is a compact nucleus from which hangs a comet-like feathery downgrowth. In our original paper (1938) we ignored these partially haemolytic Group B strains, not regarding them as true haemolytic streptococci. A colleague subsequently pointed out to us that when grown on the ordinary blood agar plate containing meat extract, there was a narrow ring of haemolysis, and they would therefore be regarded as haemolytic streptococci.

To recapitulate, the distinguishing features of these partially haemolytic Group B strains are (1) a low conical colony surrounded by an area of partial haemolysis on the peptone blood agar medium; (2) an elongated feathery comet-like colony in the serum peptone agar medium.

It is true that "F" colonies of Group A haemolytic streptococci are sometimes surrounded by a zone of partial haemolysis when grown on peptone blood agar, but the "F" colony, resembling the top of a molar tooth, is very easy to recognize on this medium. Furthermore, such strains invariably produce opalescence in the serum broth medium.

In a recent report, Miss Butler (1938) condemned this cultural test in no uncertain terms. On examining Miss Butler's results, it was clear that her main difficulty was with the Group B strains which grew as feathery colonies in the serum peptone agar medium. We regret that we did not point out this source of error in our original paper (1938) and how it could be avoided. We do not, however, agree with Miss Butler in her statement that these group B streptococci are haemolytic on the peptone blood agar medium, if "haemolytic" is used in its accepted sense as denoting a clear zone of haemolysis surrounding the colony. But however one interprets "haemolytic", the fact remains that these Group B streptococci can be recognized on the peptone blood agar medium easily enough, as can the "feathery" colonies in the serum peptone agar medium, when a little experience has been gained. We accept Miss Butler's statement that occasionally Group C and Group G strains, on fresh isolation, may show the cultural phenomena characteristic of Group A strains. In a more extensive experience with this test than Miss Butler's, we have encountered one such strain.

We ourselves are more concerned with a more serious source of error, which we have already pointed out (1938). We here refer to the occasional Group A strains which are not detected by the cultural test. On only one occasion have we succeeded in typing such a strain. This was apparently a variant of an otherwise

typical Type 8 culture, for it was agglutinated by the Type 8 serum. Our tentative theory concerning these strains is that they are variants, which have lost the ferment which denatures the serum protein, and therefore produce no opalescence in the serum broth.

In conclusion we believe, despite Miss Butler's criticism, that there is "good correlation between the information afforded by this biological test and that afforded by Lancefield's serological test", although this correlation is not perfect. Complete agreement was not, and is not, claimed. If time is no object, no one would prefer the cultural test to the serological test, which must always remain the standard test for the grouping of haemolytic streptococci. But if time is a consideration, as it must be in a routine laboratory, the cultural test will give fairly accurate results with a little experience in its use. If the interpretation of the test is in doubt, as sometimes happens, the serological test must of course be carried out.

REFERENCES.

Butler, Hildred M. (1938): Med. J. Austral., II, p. 903.
Ward, Hugh K. and Rudd, G. V. (1938): Austral. J. exp. Biol., 16, p. 181.

THE VITAMIN A AND VITAMIN D CONTENTS OF MUTTON BIRD OIL

by

WILLIAM DAVIES

(From the Department of Chemistry, University of Melbourne).

(Submitted for publication 18th November, 1938.)

Vitamin A.

During the last few years mutton bird oil (from the local petrel *Puffinus tenuirostris*) has been sold in Victoria for the same purpose as cod liver oil. The oil is obtained from the stomach of the young bird at the end of the nesting season, and it has been regarded as a reliable source of vitamin A, following the work of Carter and Malcolm (1927), who found, however, that the A content of the oil was variable. Indeed, according to Malcolm (1926), "mutton bird oil is one of the richest known natural sources of vitamin A". Rosenheim and Webster (1927) who examined the stomach oil of a very similar northern hemisphere bird, the Fulmar Petrel, claimed that the vitamin A content was approximately that of a "standard cod liver oil". These biological experiments were carried out in the very early days of vitamin A estimations before the modern physical and chemical methods were perfected, and consequently these early results cannot be expected to be accurate. Moreover, the reference to a "standard cod liver oil" as a basis of comparison has a merely qualitative and not quantitative meaning, as the vitamin A content of cod liver oils is very variable.

In view of the importance attached to mutton bird oil, the vitamin A content in various specimens has been estimated. This has been carried out by non-biological methods, namely the Carr-Price process, using the Lovibond tintometer to estimate the intensity of the blue colour in the non-saponifiable fraction, and the measurement of the intensity of absorption at 328 mμ of a solution in cyclohexane, Davies and Field (1937). Good agreement between the two methods has always been obtained.

EXPERIMENTAL.

Estimations have been made with both commercial and fresh, non-commercial samples. For the fresh specimens, more than ten young birds were caught on Babel Island[1] in April, 1938, and the stomach oil was obtained from them by

[1] By Senior Constable C. Berryman of the Tasmanian Police, to whom the author's thanks are due.

killing and "squeezing", which is the usual commercial practice. The specimens, in dark, air-tight bottles, were at once forwarded by air to the University; the oils were mixed, freed from "gurry" by filtration through glass wool, and the small amount of water separated, all these operations being carried out at ordinary temperatures. A sample of the mixed specimens was analysed for both vitamins A and D.

The results of the analysis (for A) of this and the commercial samples are summarized in Table 1. 0·001 p.c. of vitamin A (less than one Blue Unit) is the minimum amount that can be estimated by the non-biological methods, and values below this are considered as nil. 100 grams of oil were saponified in alcoholic potassium hydroxide in each case.

TABLE 1.

Source of Oil.	Non-sap. p.c.	Blue Units.	$E^{1\%}_{1\,cm.}$ 328 mμ	Percentage of A in oil calculated from Blue Units.	Percentage of A in oil calculated from $E^{1\%}_{1\,cm.}$ 328 mμ.
Commercial crude No. 1	23·1	1·5	0·03	0·002	0·002
Commercial crude No. 2	18·5	3	0·065	0·0038	0·004
Commercial refined	25·7	nil	nil	nil	nil
Commercial emulsion	10·5	nil	nil	nil	nil
Fresh non-commercial	37·0	4	0·08	0·005	0·005

It should be noted that the non-saponifiable fractions of oils rich in vitamin A always give the odour of violets on exposure to air. This is due to the oxidation of the vitamin to β-ionone and has not been noticed with any mutton bird oil in this investigation.

DISCUSSION.

Carter (1928) analysed the stomach oil and obtained the percentage of non-saponifiable matter as 38·4, which agrees well with that of the present investigation (37·0 p.c.). The physical and chemical properties of his oil and non-saponifiable fraction agree closely with those of the oil from Babel Island, and indicate that the oils are practically identical. Carter's detailed analysis shows that there is only a trace of glycerides or true fat present, and that the unusually large non-saponifiable fraction (which is several times greater than that found in most local fish-liver oils), consists chiefly of cetyl alcohol (65 p.c.), oleyl alcohol (28 p.c.) and cholesterol (7 p.c.). Mutton bird oil is therefore not a true fat, but almost entirely a liquid wax, like sperm oil. It closely resembles the stomach oil of the Fulmar Petrel which Rosenheim and Webster (loc. cit., p. 116) suggest represents "the indigestible residue of fatty foodstuffs". It is also suggested that the oil is used for preening purposes and to protect the feathers from sea water. It is very probable that the stomach oil of the mutton bird has a similar origin and function.

Though Carter and Malcolm (loc. cit.) have shown that a considerable proportion of mutton bird oil can be absorbed, yet a large proportion of the oil passes through apparently unchanged and is consequently useless from the nutritional point of view. From the present investigation it is clear that the oil is also virtually useless as a source of vitamin A, even before it has been subjected to the refining processes which destroy the vitamin. Jowett and Davies (1938) have shown that the liver oils of common local fish, such as the snapper shark and barracouta, usually contain between 0·5 p.c. and 4 p.c. of vitamin A, that is 100 to 800 times the concentration in mutton bird oil. It would therefore be much better to exploit these oils as a vehicle for vitamin A than to continue the use of mutton bird oil.

Vitamin D.

The mixed sample of the oils from Babel Island was assayed for vitamin D by the prophylactic biological method of Hume, Pickersgill, and Gaffikin (1932), using albino rats. The details of the method of estimation are given by Cunningham (1935). The author is indebted for the assay to Dr. M. M. Cunningham, of the Veterinary Laboratory, Department of Agriculture, Wallaceville, New Zealand, who reports that the mutton bird oil contains the "very low value of five international units per gram".

Mutton bird oil as a vehicle for vitamin D cannot be compared with some common oils such as cod and halibut liver oils, which have 60 to 300 and 200 to 4,000 international units per gram respectively (Fixsen and Roscoe, 1938). It is noteworthy in this connection that the liver oil (abundant in vitamin A) of the groper, a common New Zealand fish, contains 2,250 to 2,300 international units per gram (Cunningham, loc. cit.) and that therefore a local medicinal oil rich both in D and A may be available if need arose.

SUMMARY.

The vitamin A content of mutton bird oil is of the very low order of 0·005 p.c. (four Blue Units), commercial unrefined specimens containing rather less than this. "Refined" and commercial emulsions of mutton bird oil contain no measurable quantity of vitamin A, the processes or chemicals introduced having destroyed the vitamin.

Vitamin D is also present in almost negligible quantity (five international units per gram).

Finally, mutton bird oil is of little use as a food, as it is mainly a liquid wax rather than a fat.

ACKNOWLEDGMENTS.

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REFERENCES.

Carter, C. L. (1928): J. Soc. chem. Ind., 47, p. 26T.
Carter, C. L. and Malcolm, J. (1927): Biochem. J., 21, p. 484.
Cunningham, M. M. (1935): J. of Science and Technology, New Zealand, 17, p. 563.
Davies, W. and Field, D. J. (1937): Biochem. J., 31, p. 248.
Fixsen, M. A. B. and Roscoe, M. H. (1938): Nutrition Abst. and Rev., 7, pp. 860, 861.
Hume, E. M., Pickersgill, M. and Gaffikin, M. M. (1932): Biochem. J., 26, p. 488.
Jowett, W. G. and Davies, W. (1938): Commonwealth Council Sci. Ind. Res., Pamphlet No. 85, p. 28.
Malcolm, J. (1926): Trans. New Zealand Institute, 56, p. 658.
Rosenheim, O. and Webster, T. A. (1927): Biochem. J., 21, p. 114.

THE SEROLOGICAL RELATIONSHIP OF MYXOMA AND SHOPE'S FIBROMA VIRUSES

by

DORA LUSH[1]

(From the Walter and Eliza Hall Institute, Melbourne).

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Since Shope showed that rabbits recovered from an infection with his fibroma virus (Shope, 1932) were relatively immune to infection with myxoma, the exact relationship of these viruses has attracted the attention of numerous workers, with somewhat conflicting results. Shope failed to find evidence of *in vitro* inactivation of myxoma virus with fibroma antiserum, the rabbits inoculated with serum-virus mixtures developing typical myxomatosis. Rivers and Ward (1937) showed that the serum of rabbits recovering from infection with Shope's fibroma virus contains agglutinins for the washed elementary bodies of myxoma and precipitins for the non-infective soluble substance present in Seitz filtrates of emulsions from infected skin, or of the serum of animals acutely ill with myxoma. The presence of cross agglutinins has also been described by Ledingham (1937). Complement fixation has been tested by van Rooyen and Rhodes (1938), who obtained no cross reactions and concluded that this test was more specific than agglutination or precipitation.

The work described in this paper is concerned only with the *in vitro* inactivation of myxoma virus with fibroma antiserum, the results of experiments with myxoma virus and homologous antiserum having been discussed elsewhere (Lush, 1937); and with the complement fixation test using myxoma antigens and both types of antiserum.

The Inactivation of Myxoma Virus by Fibroma Antiserum.

Serum-virus mixtures were tested by two methods (1) the inoculation of rabbits, and (2) the inoculation of the chorioallantoic membrane of the developing egg, following the technique described by Burnet (1936) and used extensively in this laboratory.

Technique. The myxoma virus was obtained from Dr. L. B. Bull and has been maintained without change in pathogenicity by serial passage on the chorioallantoic membrane. The fibroma virus OA strain (Andrewes, 1936) was supplied by Dr. E. Weston Hurst and was maintained by intratesticular inoculation in rabbits. Serum was obtained from rabbits D.98 and D.102, which had reacted well to simultaneous subcutaneous and intratesticular inoculation of fibroma virus

[1] Work carried out under grants for research on virus diseases from the Rockefeller Foundation and from the National Health and Medical Research Council.

and had shown no reaction to a further intratesticular inoculation four weeks later. Blood was taken five weeks after the second inoculation. Pure myxoma antiserum was obtained from two rabbits which had recovered after a typical attack of myxomatosis induced in both instances by contact infection. They were given a subcutaneous inoculation during convalescence and were bled three weeks later (MC.1, KC.5). Serum-virus mixtures were kept in an ice bath for 1–1½ hours before the inoculation of eggs and rabbits. The eggs were inoculated with 0·05 c.c. and the rabbits received 0·2 c.c. intradermally.

TABLE 1.

Inactivation of Myxoma Virus by Anti-Fibroma Serum D.102.

Serum.	Myxoma Virus dilution.	Rabbits 0·2 c.c. intradermal.	Egg membrane 0·05 c.c. Average count 4–6 membranes.	p.c. Survival.
Nil	1: 10^6	S, S, +(7)		
	1: 10^5	+(7), +(7), +(7)		
	1: 10^4	+(7), +(7), +(7)		
	1: 10^3		11·8	
	1: 300		33·5	
D.102 1: 2	1: 2		42·4	0·7%
	1: 20		10·0	1·6%
	1: 200	+(7), +(7), +(7)		
	1: 2,000	+(7), +(9), +(7)		
	1: 20,000	S, S, +(8)		2·0%

S = survival without local lesion.
\+ = local lesion followed by generalization with death on the day shown in brackets.

It is clear from the results in Table 1 that the serum from rabbit D.102 was capable of inactivating myxoma virus by both methods employed. The fibroma antiserum from rabbit D.98 gave an entirely similar result on the chorioallantois and was not tested in rabbits. Cross tests with myxoma antiserum and fibroma virus were not attempted as fibroma virus is of low titre in the rabbit, and will not multiply on the developing egg membrane.

Complement Fixation.

Two different types of antigen were used in these experiments, one, the usual infective virus emulsions of myxoma and a control virus, usually influenza or herpes, prepared from egg membrane lesions, and the other a Seitz filtrate of the myxoma emulsions containing the soluble substance described by Rivers and Ward (1937). The control antigen was always used, as it was found that rabbits' sera in low dilutions tended to be anti-complementary.

Technique. Antigens: These were prepared from two or three day egg membranes showing confluent lesions which were washed in saline, ground with quartz powder and emulsified in a small volume (about 1 c.c. per membrane) of saline. The emulsions were spun in a small angle centrifuge (3,000 r.p.m.) for 30 minutes and the supernatant fluid used as antigen. The soluble substance was prepared in the same way, filtered through a Seitz E.K. pad and tested to make sure it was non-infective. Merthiolate 1: 20,000 was then usually added. *Sera:* All sera were diluted 1: 5 or 1: 10 and freshly inactivated before use (56°C. for 20 minutes). *Complement:* Fresh guinea-pig serum was titrated each day and a dilution made so that 0·1 c.c. (two drops

from standard pipette) contained 3 M.H.D. *Sensitized Red Cells:* The test dose consisted of 0·1 c.c. of a sensitized 3 per cent. suspension of sheep erythrocytes containing 5 M.H.D. of haemolytic amboceptor per c.c. *The Test:* Doubling dilutions of each serum were prepared and the test set up as follows: saline 0·1 c.c. (two drops), serum 0·1 c.c., complement 0·1 c.c. and antigen 0·1 c.c. The reagents were added to the tubes in the order given, mixed thoroughly by shaking and placed in the ice box over night (usually 18 hours). The sensitized cells were then added, the mixtures shaken, placed in a water bath at 37°C. for half-an-hour and shaken frequently. The results were read immediately after removal from the water bath. Controls always included the lowest dilution of antigen used with 1½ M.H.D. complement and 1½ M.H.D. complement with saline. Rabbit serum was frequently found to be anticomplementary in the lower dilutions. In Table 2 the lowest dilution of serum used in each case was the lowest dilution which did not inhibit haemolysis with 1½ M.H.D. complement.

TABLE 2.

Complement Fixation using 3 M.H.D. Complement.

Serum.	Antigen.	Serum dilutions. 1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560
KC.5 (myxoma antiserum)	Myxoma (unfiltered) diluted 1:2		+	+	+	+	+	+	+	−
	1:5		+	+	+	+	+	+	+	−
	1:20		+	+	+	+	+	+	−	−
	Myxoma S.S. (Seitz filtrate) undiluted		+	+	+	+	+	+	±	−
	diluted 1:5		+	+	+	+	+	±	−	−
	Herpes control undiluted		±	−	−	−	−	−	−	−
D.102 (fibroma antiserum)	Myxoma undiluted	+	+	+	+	−				
	diluted 1:2	+	+	+	−	−				
	1:5	+	±	−	−	−				
	Myxoma S.S. undiluted	−	−	−	−	−				
	diluted 1:5	−	−	−	−	−				
	Herpes control undiluted	−	−	−	−	−				

+ = No lysis of RBC's. − = complete lysis of RBC's. Sera from rabbits MC.1 (myxoma) and D.98 (fibroma) gave results similar to those given in the Table, but were not tested with the soluble substance.

DISCUSSION.

Fibroma immune rabbits are not completely immune to myxoma virus, but the infection is greatly modified and nearly always non-fatal. *A priori* one would expect that their serum would be capable of inactivating a certain amount of myxoma virus, but not so effectively as myxoma antiserum. The results reported in the first section of this paper show that this expectation is fulfilled if graded small doses of virus are used. There is no essential discrepancy between this result and Shope's failure to observe inactivation of myxoma virus by antifibroma serum, since he used only a single, rather large dose of myxoma virus in his tests. It has been suggested by Ledingham (1937) that the relative immunity of fibroma

rabbits to myxoma is to be related to the agglutinins for elementary bodies of myxoma present in their serum, and not to virus inactivating antibody. This suggestion that agglutinin and inactivating antibody are distinct may be correct, but all our results are more simply interpreted on the orthodox view that agglutination, complement fixation and virus-inactivation are all manifestations of one antigen-antibody reaction, and that the antigenic relationship of fibroma and myxoma viruses is of the type familiar to every systematic bacteriologist who has to deal with serologically related but not identical bacteria.

The complement fixation results in Table 2 offer some indication that the soluble antigen in the Seitz filtrate reacts more specifically than the original emulsion containing elementary bodies as well. In view of Rivers and Ward's results with precipitin tests it seems more likely, however, that the difference is due to the lower concentration of antigen in the filtrate. A comparison of the reactions of the myxoma immune serum KC.5 and the fibroma serum D.102 will show that higher concentrations of antigen are necessary to give optimal fixation with the fibroma serum than with myxoma serum. Similar quantitative considerations may be responsible for the difference between our results and those of van Rooyen and Rhodes. Their failure to demonstrate complement fixation with antifibroma serum and myxoma antigen may well have been due simply to the use of too small a concentration of antigen.

SUMMARY.

1. Inactivation *in vitro* of myxoma virus by fibroma antiserum has been demonstrated by tests on the chorioallantoic membrane of the developing egg and in the rabbit.

2. Fixation of complement was obtained using myxoma antigen with both myxoma and fibroma antisera.

3. Fixation of complement was obtained using non-infective Seitz filtrates of myxoma virus emulsions with myxoma antiserum but not with a fibroma antiserum.

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REFERENCES.

Andrewes, C. H. (1936): J. exp. Med., 63, p. 157.
Burnet, F. M. (1936): Sp. Rep. Ser. Med. Res. Coun. Lond., No. 220.
Ledingham, J. C. G. (1937): Brit. J. exp. Path., 18, p. 436.
Lush, Dora (1937): Austral. J. exp. Biol., 15, p. 131
Rivers, T. M. and Ward, S. M. (1937): J. exp. Med., 66, p. 1.
van Rooyen, C. E. and Rhodes, A. J. (1938): Zbl. Bakt. I Orig., 142, p. 149.
Shope, R. E. (1932): J. exp. Med., 56, p. 803.

THE ASH CONSTITUENTS OF AUSTRALIAN FISH

by

F. W. CLEMENTS AND R. C. HUTCHINSON

(From the Australian Institute of Anatomy, Canberra, A.C.T.).

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Australia possesses a very abundant and varied fish fauna which embraces both temperate and tropical varieties. Most of the fish are edible, and about fifty kinds are to be bought in the open market. Australians do not consume as much fish as might be expected when one considers that a large percentage of the population lives close to the sea. Data on fish consumption are difficult to obtain because large quantities are caught and consumed without passing through the regular channels of trade. From data recently obtained by the Commonwealth Advisory Council on Nutrition it has been possible to draw up Table 1, comparing the fish and meat consumption in all states except Tasmania.

TABLE 1.

Food.	N.S.W.	Vic.	Q.	S.A.	W.A.	Average.
Fish (Fresh and Canned)						
Lb. consumed weekly per adult male	·36	·32	·43	·26	·34	·34
Pence spent weekly per adult male	4·36	3·41	4·60	3·13	3·93	3·89
Meat (Fresh and Canned)						
Lb. consumed weekly per adult male	3·75	4·37	3·67	3·53	3·40	3·75
Pence spent weekly per adult male	27·66	31·05	29·46	26·21	26·83	28·24

The adult male unit is that recommended by an expert committee of the League of Nations (1938).

The moisture, ash and ash constituents in the edible portion of all those varieties of Australian fish, mollusca and crustacea which can be bought in the open market, have been estimated. No attempt has been made to include the small and varied assortment of fish which is caught by the amateur fisherman. The ash constituents appear to vary slightly according to the locality in which the fish, mollusca or crustacea, are found. This is particularly noticeable in the case of oysters and mussels. The majority of the results given in Table 3 are from the analyses of several samples of fish; and, where possible, samples were collected from Queensland, New South Wales, Victoria, Tasmania and South Australia. The fish used in these analyses were purchased in fish shops and markets. In those instances where only one sample was analysed the results given would be within the neighbourhood of the true value.

TABLE 3.

Mineral Constituents Expressed as Percentage of Edible Portion.

Common Name.	Scientific Name.	% Moisture.	% Ash.	% K	% P	% Na	% Mg	% Ca	% Fe	% Cu	% Mn	Number of Samples	Ca P
Barracouta	*Thyrsites atun*	85	1·8	·36	·27	·08	·04	·07	·001	1×10^{-5}	4×10^{-7}	2	·26
Barramundi	*Lates calcarifer*	78	1·4	·29	·15	·07	·02	·03	·001	2×10^{-6}	1×10^{-6}	1	·22
Bay Trout	*Arripis trutta*	76	1·2	·23	·15	·06	·02	·03	·001	1×10^{-5}	2×10^{-7}	3	·18
Blackfish	*Girella tricuspidata*	76	1·4	·31	·29	·05	·02	·04	·001	3×10^{-6}	trace	1	·14
Bream	*Roughleyia australis*	73	1·0	·23	·18	·04	·02	·02	·001	3×10^{-6}	,,	2	·13
Butterfish	*Scatophagus argus*	78	1·8	·37	·28	·09	·04	·06	·002	1×10^{-5}	,,	3	·22
Conger Eel	*Leptocephalus* sp.	77	1·0	·20	·22	·05	·02	·02	·001	1×10^{-6}	,,	1	·09
Coral Cod	*Epinephelus* sp.	82	1·2	·24	·20	·05	·04	·03	·001	2×10^{-6}	,,	1	·13
Crab	*Portunus pelagicus*	79	2·6	·52	·22	·12	·13	·14	·002	1×10^{-5}	,,	5	·62
Crayfish	*Jasus lalandii*	75	1·2	·24	·27	·06	·04	·04	·001	1×10^{-5}	,,	2	·13
Flathead	*Neoplatycephalus macrodon*	83	1·0	·19	·26	·04	·01	·03	·001	3×10^{-6}	,,	6	·12
Flake	*Mustelus antarcticus*	75	1·4	·29	·16	·07	·01	·03	·001	4×10^{-6}	,,	1	·20
Flounder	*Pleuronectes arsius*	81	1·1	·26	·16	·06	·01	·03	·001	1×10^{-5}	,,	2	·13
Garfish	*Reporhamphus australis*	78	1·2	·25	·16	·07	·02	·03	·001	2×10^{-6}	,,	12	·18
Groper	*Epinephelus ergastularius*	76	1·4	·28	·15	·06	·01	·02	·001	1×10^{-5}	3×10^{-7}	2	·14
Herring	*Potamalosa novae-hollandiae*	71	1·4	·29	·30	·07	·03	·05	·001	1×10^{-5}	trace	6	·15
Jewfish	*Sciaena antarctica*	79	1·2	·26	·17	·07	·03	·04	·001	1×10^{-5}	,,	1	·26
John Dory	*Zeus australis*	77	1·3	·33	·13	·06	·02	·04	·001	2×10^{-6}	,,	2	·33
King Fish	*Regificola grandis*	75	1·4	·28	·24	·09	·04	·03	·001	1×10^{-5}	,,	1	·13
Leather Jacket	*Monacanthus chinensis*	80	0·8	·19	·13	·03	·02	·04	·001	4×10^{-6}	,,	2	·31
Mackerel	*Scomber australasicus*	73	1·6	·42	·27	·10	·04	·02	·001	3×10^{-6}	,,	3	·18
Mullet	*Mugil cephalus*	65	1·6	·32	·17	·10	·03	·03	·001	3×10^{-6}	,,	3	·20
Murray Cod	*Maccullochella macquariensis*	83	1·2	·24	·11	·06	·02	·03	·001	1×10^{-5}	,,	1	·22
Murray Perch	*Plectropcites ambiguus*	78	1·2	·21	·13	·08	·02	·03	·001	4×10^{-6}	,,	1	·22
Mussel	*Mytilus obscurus*	86	1·6	·38	·17	·08	·02	·08	·001	2×10^{-5}	8×10^{-7}	24	·49
Nannygai	*Trachichthodes affinis*	79	1·1	·21	·12	·10	·02	·03	·001	2×10^{-6}	trace	2	·27
Oyster	*Saxostrea commercialis*	84	2·3	·57	·15	·16	·05	·09	·005	3×10^{-5}	,,	60	·63
Perch	*Cheilodactylus macropterus*	77	1·4	·32	·13	·08	·02	·04	·001	1×10^{-5}	,,	2	·33
Prawn	*Penaeus* sp.	78	2·2	·38	·15	·10	·05	·04	·001	1×10^{-5}	,,	36	·30
Rock Cod	*Roboralga jacksoniensis*	80	1·3	·27	·21	·07	·02	·02	·001	1×10^{-5}	,,	2	·13
Salmon Trout	*Arripis trutta*	76	1·2	·24	·16	·06	·03	·04	·001	1×10^{-5}	,,	4	·24
Sea Salmon	*Polynemus* sp.	76	1·2	·24	·16	·06	·03	·02	·001	1×10^{-5}	,,	4	·10
Shrimp	——	74	2·4	·41	·22	·11	·04	·05	·002	1×10^{-5}	,,	24	·22
Scallop	*Pecten fumatus*	79	1·8	·34	·26	·20	·02	·04	·001	1×10^{-5}	1×10^{-6}	100	·16
Skate	(A species of *Raja*)	78	1·4	·25	·17	·09	·03	·07	·001	1×10^{-5}	trace	1	·40
Skipjack or Tailor	*Pomatomus pedica*	69	1·4	·27	·18	·06	·03	·03	·001	1×10^{-5}	,,	3	·16
Snapper	*Chrysophrys guttulatus*	65	1·8	·40	·16	·08	·03	·02	·001	1×10^{-5}	,,	2	·12
Snook	*Sphyraena novae-hollandiae*	77	1·2	·29	·12	·08	·03	·04	·001	2×10^{-6}	,,	6	·31
Sole	*Synaptura nigra*	75	1·5	·23	·23	·08	·03	·03	·001	3×10^{-6}	,,	2	·15
Tommy Rough	*Arripis georgianus*	71	1·2	·25	·24	·05	·03	·04	·001	3×10^{-6}	2×10^{-7}	6	·15
Trumpeter	*Pelates sexlineatus*	71	1·0	·20	·11	·07	·01	·02	·001	4×10^{-6}	trace	1	·23
Teraglin	*Cynoscion atelodus*	70	1·2	·28	·12	·06	·03	·04	·001	1×10^{-5}	,,	1	·31
Trevally	*Caranx georgianus*	74	1·5	·34	·15	·07	·03	·04	·001	4×10^{-6}	,,	1	·22
Whiting	*Sillago* sp.	78	1·1	·26	·13	·05	·02	·03	·001	1×10^{-5}	,,	8	·21
Yellow Jacket	*Pseudomonacanthus ayraudi*	76	1·4	·34	·13	·07	·03	·03	·001	4×10^{-6}	,,	2	·25

Methods of Analysis.

Moisture was determined by the toluene distillation method [Official and Tentative Methods of Analysis (1935a)]. Five grams of fish were taken for each ash determination. When drying off the remainder of the fish, a little alcohol was added to assist saponification. Extreme care was necessary in ashing, and generally the sample had to be mixed with water, dried and incinerated several times to obtain a satisfactory ash. In order to estimate all ash constituents, except sulphur and manganese, from five to ten grams of ash were dissolved in the minimum amount of hydrochloric acid, and the volume made up to 100 c.c. with water. (Occasionally there was a slight residue which had to be filtered off and for which allowance had to be made.)

The mineral elements were estimated by methods similar to those outlined by Morris and Rosedale (1935), with the exception of sodium, potassium, sulphur and magnesium. Magnesium was precipitated in the filtrate from the calcium estimation with 8-hydroxyquinoline according to the method of Mitchell and Ward (1932). The mixed chlorides of sodium and potassium were determined in the filtrate from the calcium and magnesium estimations and potassium then determined by the perchlorate method as potassium perchlorate. Sodium was found by difference. To determine sulphur a portion of the dried fish was treated with concentrated magnesium nitrate before ashing and the estimation carried out as described in "Official and Tentative Methods of Analysis" (1935b).

From the results a table has been prepared to show the mean mineral composition of Australian fish (Table 2).

TABLE 2.

Mean Mineral Composition of Australian Fish.

Element.	P.c. in Ash.	P.c. in Edible Portion.
Potassium	20·97	·29
Sulphur	13·81	·19
Phosphorus	13·65	·19
Sodium	5·33	·07
Magnesium	1·91	·03
Calcium	2·79	·04
Iron	·071	·001
Copper	·0004	·00001
Manganese	·00004	·0000006

A fish consists of 3 to 20 p.c. skin, and this has an average ash content of about 3 p.c. In all cases where the skin is eaten, the adhering skin has been analysed together with the flesh, thus giving a slightly different ash content than would be obtained if the flesh alone were analysed. The edible portion of Australian fish contains approximately 1·4 p.c. of ash, which is slightly more than the muscle tissue of beef or mutton, but less than that of dried beans, peas or lentils. The average moisture content is 77 p.c.

During the present set of analyses the non-metal sulphur was not estimated for all fish, but the average figure for five determinations carried out on samples of flathead, barracouta, mullet, whiting and yellow jacket have been included in Table 2.

It is difficult to compare the results obtained in this survey with figures given for overseas fish, for many Australian fish do not appear in overseas lists, and totally different species often have the same common name in different countries. Also many of the figures are from the analysis of a single specimen, and it has been found that individual variation is often considerable.

The calcium/phosphorus ratio has been computed for each fish, and those for which the ratio is high (that is, greater than 0·3) are: crab, John Dory, leather jacket, mussel, oyster, perch, prawn, skate, snook, and teraglin; and those for which it is low (that is less than 0·14) are: blackfish, bream, conger eel, coral cod, crayfish, flathead, flounder, kingfish, rock cod, sea salmon and snapper.

SUMMARY.

The moisture, ash, potassium, phosphorus, sodium, magnesium, calcium, iron, copper and manganese contents of forty-five varieties of Australian fish have been determined.

Some data on the consumption of fish in Australia are presented.

REFERENCES.

Association of Official Agricultural Chemists (1935): "Official and Tentative Methods of Analysis" (a, p. 335: b, p. 321).

League of Nations (1938): Quarterly Bulletin of the Health Organizations, 4, p. 323.

Mitchell, A. D. and Ward, A. M. (1932): "Modern Methods of Quantitative Analysis".

Morris, J. P. and Rosedale, J. L. (1935): Malayan Med. J., 10, p. 1.